

# The compositional and evolutionary logic of metabolism

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(Dated: July 25, 2012)

Metabolism is built on a foundation of organic chemistry, and employs structures and interactions at many scales. Despite these sources of complexity, metabolism also displays striking and robust regularities in the forms of modularity and hierarchy, which may be described compactly in terms of relatively few principles of composition. These regularities render metabolic architecture comprehensible as a system, and also suggests the order in which layers of that system came into existence. In addition metabolism also serves as a foundational layer in other hierarchies, up to at least the levels of cellular integration including bioenergetics and molecular replication, and trophic ecology. The recapitulation of patterns first seen in metabolism, in these higher levels, motivates us to interpret metabolism as a source of causation or constraint on many forms of organization in the biosphere. Many of the forms of modularity and hierarchy exhibited by metabolism are readily interpreted as stages in the emergence of catalytic control by living systems over organic chemistry, sometimes recapitulating or incorporating geochemical mechanisms.

We identify as modules, either subsets of chemicals and reactions, or subsets of functions, that are re-used in many contexts with a conserved internal structure. At the small molecule substrate level, module boundaries are often associated with the most complex reaction mechanisms, catalyzed by highly conserved enzymes. Cofactors form a biosynthetically and functionally distinctive control layer over the small-molecule substrate. The most complex members among the cofactors are often associated with the reactions at module boundaries in the substrate networks, while simpler cofactors participate in widely generalized reactions. The highly tuned chemical structures of cofactors (sometimes exploiting distinctive properties of the elements of the periodic table) thereby act as “keys” that incorporate classes of organic reactions within biochemistry.

Module boundaries provide the interfaces where change is concentrated, when we catalogue extant diversity of metabolic phenotypes. The same modules that organize the compositional diversity of metabolism are argued, with many explicit examples, to have governed long-term evolution. Early evolution of core metabolism, and especially of carbon-fixation, appears to have required very few innovations, and to have used few rules of composition of conserved modules, to produce adaptations to simple chemical or energetic differences of environment without diverse solutions and without historical contingency. We demonstrate these features of metabolism at each of several levels of hierarchy, beginning with the small-molecule metabolic substrate and network architecture, continuing with cofactors and key conserved reactions, and culminating in the aggregation of multiple diverse physical and biochemical processes in cells.

## I. INTRODUCTION

The chemistry of life is distinguished by being both highly ordered and far from thermodynamic equilibrium [1].<sup>1</sup> This dynamical order is maintained by the non-equilibrium transfer of electrons through the biosphere. Free energy from potential differences between electron donors and acceptors can be derived from a variety of biogeochemical cycles [4], but within cells electron transfer is mediated by a small number of universal electron carriers which drive a limited array of organic reactions [5]. Together these reactions make up metabolism, which governs the chemical dynamics both within organ-

isms and across ecosystems. The universal and apparently conserved metabolic network transcends all known species diversification and evolutionary change [6, 7], and distinguishes the biosphere within the major classes of planetary processes.<sup>2</sup> We identify metabolism with the quite specific substrate architecture and hierarchical control flow of this network, which provide the most essential characterization of the chemical nature of the living state.

Understanding the structure of metabolism is central to understanding how physics and chemistry constrain life and evolution. The polymerization of monomers into selected functional macromolecules, and the even more complex integration and replication of complete cells, form a well-recognized hierarchy of coordination and information-carrying processes. However, in the sequence of biosynthesis these processes come late, and they involve a much smaller and simpler set of chemical

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<sup>1</sup> Applying the often-invoked term “far from equilibrium” to biochemistry requires care. When catalysts (including transporters or other molecular machinery) create a separation of timescales between supported reactions and autonomous parasitic reactions, the supported reactions can sometimes be treated as an isolated subsystem with equilibrium approximations [2, 3], though the isolation itself is a cumulative deviation far from equilibrium.

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<sup>2</sup> Ref. [8] first proposed classifying the biosphere as the fourth “geosphere”, parallel to the lithosphere, hydrosphere, and atmosphere that have provided a classical taxonomy in geology.

reactions than core metabolism, the network in which all basic monomer components of biomass are created from environmental inputs. Because the core is the origin of all biomass, its flux is perforce higher than that in any secondary process; only membrane electron transport (reviewed in Ref. [4]) has higher energy flux.<sup>3</sup> Metabolism is the sub-space of organic chemistry over which life has gained catalytic control. Because in the construction and optimization of biological phenotypes all matter flows through this sub-space, its internal structure imposes a strong filter on evolution.

In this review we identify a number of organizing principles behind the major universal structures and functions of metabolism. They provide a simple characterization of metabolic architecture, particularly in relation to microbial metabolism, ecology, and phylogeny, and the major (biogeochemical) transitions in evolution. We often find the same patterns of organization recapitulated at multiple scales of time, size, or complexity, and can trace these to specific underlying chemistry, network topology, or robustness mechanisms. Acting as constraints and sources of adaptive variation, they have governed the evolution of metabolism since the earliest cells, and some of them may have governed its emergence. They allow us to make plausible reconstructions of the history of metabolic innovations and also to explain certain strong evolutionary convergences and the long-term persistence of the core components of metabolic architecture.

Many structural motifs in both the substrate and control levels of metabolism may be interpreted as functional modules. By isolating effects of perturbation and error, modularity can both facilitate emergence, and support robust function, of hierarchical complex systems [10, 11]. It may also affect the large-scale structure of evolution by favoring variation in the regulation and linkage between modules, while conserving and thereby minimizing disruption of their internal architecture and stability [12, 13]. This can enhance evolvability through two separate effects. An increased *phenotypic* (i.e. structural or functional, as opposed to *genotypic* or sequence) robustness of individual modules gives access to larger genetic neutral spaces and thus a greater number of novel phenotypes at the boundaries of these spaces [14]. At the same time, concentrating change at module interfaces, and allowing combinatorial variation at the module level, can decrease the amount of genetic variation needed to generate heritable changes in aggregate phenotypes [15, 16]. It has been argued that asymmetries in evolutionary constraints can be amplified through direct selection for evolvability, and that this is a central source of modularity and hierarchy within biological sys-

tems [15–18].

These functional consequences of modularity lead us to expect that intermediary metabolism will be modular as a reflection of the requirements of emergence and internal stability. Certainly we observe this empirically; many topological analyses of metabolic networks find a modular and hierarchical structure [19–21]. We also expect that, with the numerous and diverse constraints from chemistry and physics in core metabolism, and their large impact on metabolic flux, the evolutionary consequences of chemical modularity will be greatest from the core, and will diminish as chemical mechanisms are simplified, and the impact on flux is reduced, in more peripheral stages of biosynthesis.

To understand the origin and evolutionary consequences of modularity in metabolism, however, we will need system-level representations that go beyond topology, to include sometimes quite particular distinctions of function. Details of substrate chemistry, enzyme grouping and conservation, and phylogenies of metabolic modules, in particular, are rich sources of functional information and context. These will enable us to reconstruct steps in metabolic evolution and identify their environmental drivers.

#### A. Hierarchy in metabolism, and the role of individuals and ecosystems

While most metabolic conversions are performed within cells,<sup>4</sup> the structure of metabolism spans many levels of biological organization. The causes and roles of evolutionary changes, even though they arise within cellular lineages, may be only partly explained by organization at the cellular or species level. Other levels that must also be considered include the meta-metabolome of trophic ecosystems [22–24], and the links to geochemistry [25–32]. The great biochemical cycles – of carbon, nitrogen, phosphorus, or many metals – combine physiological, ecological, and even geochemical links such as mantle convection or continental weathering. The deepest universal features of metabolism are reliably seen at the ecosystem level [7, 33] but not necessarily within organisms [34].

These observations could be summarized as showing that individuality is a derived characteristic of living systems within a larger framework of metabolic regularity, a perspective that fits well with the modern understanding that individuality takes many forms which must be explained within their contexts [35]. Alternatively, in more conventional genetic descriptions of evolution [36, 37], metabolic completeness, trophic as well as physiological flux balance, and network-level response to fluctuations

<sup>3</sup> Ref. [9] notes that, over a broad sample of enzymes collected from the literature, those for secondary metabolic reactions have rates  $\sim 1/30$  the typical rates of enzymes for core reactions.

<sup>4</sup> Exceptions include siderophores and secreted enzymes, most often used at the cell-population level.

are explicit features contributing to an organism’s fitness within a co-evolving or constructed environment [38].

We can to a considerable extent disentangle the inherent chemical hierarchy of metabolism from the evolutionary hierarchy of species by studying variations in the anabolic (biosynthetic) versus catabolic (degradative) pathways within organisms, along with the relations of autotrophy (self-feeding) versus heterotrophy (feeding from others) in the ecological roles of species. We can argue for the existence of a universal anabolic, autotrophic network [39, 40] that comprises the chemistry essential to life. We can then separate the structural requirements and evolutionary history of the universal network from secondary complexities, which we will argue originate in the diversification of species and the concurrent processes of assembly of ecological communities.

Within the universal (and apparently essential) network we may identify further layers, with distinct functions and plausibly distinct origins. A functioning metabolism is both a network of fluxes through substrate molecules, and a set of hierarchical relations in which some of the more complex structures control the kinetics of flows within the network. Within the substrate network, distinguishable subnetworks include the core network to synthesize CHO backbones, networks radiating from the core that incorporate N, S, P, or metals, higher-order networks that assemble complex organics from “building blocks”, and still others that synthesize all forms of polymers from small organic monomers. Within the control hierarchy, the layers of cofactors, oligomer catalysts, and integrated cellular energetic and biosynthetic subsystems are qualitatively distinct.

The foundation of autotrophy – and more generally the anchor that embeds the biosphere within geochemistry – is *carbon-fixation*, the transformation of  $\text{CO}_2$  into small organic molecules (see Fig. 1). A recent study [41] combining evidence from phylogeny and metabolic network reconstruction<sup>5</sup> showed that all carbon fixation phenotypes may be related by an evolutionary tree with very high (nearly perfect) parsimony, and a novel but sensible phenotype at the root. The branches representing innovations in carbon fixation were found to trace the standard deep divergences of bacteria and archaea. More striking, likely environmental drivers could be identified for most divergences, suggesting that deep evolution reflects first incursions into novel geochemical environments. The tight coupling of the reconstructed phylogeny to geochemical variety suggests that constraints from chemistry and energetics drove early evolution in predictable ways, leaving little need to invoke historical contingency.

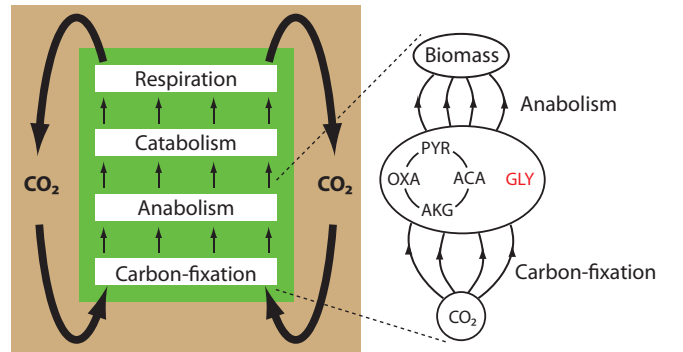


FIG. 1: The metabolic structure of the biosphere. The biosphere as a whole can be described as implementing a global biological carbon cycle based on  $\text{CO}_2$ , with carbon-fixation as the metabolic foundation. The small organic molecules produced during fixation of  $\text{CO}_2$  are subsequently transformed and built up into the full diversity of known biomolecules through the process of anabolism, before ultimately being broken back down through catabolism and re-released as  $\text{CO}_2$  through respiration. The striking modularity of metabolism is expressed in the fact that the interface between carbon-fixation and anabolism consists of a very small number of small organic molecules (shown schematically at right-center). The key observation that in addition to intermediates in the citric acid cycle – from which nearly all anabolic pathways emanate [7] (see Fig. 6) – glycine (red) should be included in this set allows a complete reconstruction of the evolutionary history of carbon-fixation [41] (see also Figures 10 and 11). Abbreviations: Acetyl-CoA (ACA); Pyruvate (PYR); Oxaloacetate (OXA);  $\alpha$ -Ketoglutarate (AKG); Glycine (GLY).

## B. Catalytic control and origins of modularity in metabolism

While carbon-fixation draws on all levels of biological organization (requiring integration and control of many cellular components), evolution in the network of its small-molecule substrate has consisted only of changes in the use of a small number of clearly defined reaction sequences. The disruption, disconnection, or reversal of these modules accounts for the full diversity of modern carbon-fixation. As we will show below, the module structure is further defined by a distinction between two types of chemistry. Within modules, the reactions are mainly (de-)hydration or (de-)hydrogenation reactions, catalyzed by enzymes from common and highly-diversified families. Module interfaces are created (and distinguished) by key carboxylation reactions, catalyzed by highly conserved enzymes, often involving special metal centers and/or complex organic cofactors. The congruence of phylogenetic branching with topological and chemical module boundaries suggests that a very small number of catalytic innovations were the key bottlenecks to evolutionary diversification, against a background of facile and readily re-used organic chemistry.

Topological modularity in the small-molecule substrate network is often associated with functional divisions in

<sup>5</sup> We refer to this approach as “phylometabolic” reconstruction.

the more complex molecules that control metabolism, particularly the cofactors, showing that their metabolic role is also an evolutionary role. As carriers of electrons or essential functional groups, cofactors regulate kinetic bottlenecks in metabolic networks. The appearance and diversification of families of biosynthetically related cofactors introduced functions which served as “keys” to domains in organic chemistry, incorporating these within biochemistry. Often we may map biosynthetic pathway diversification of cofactors onto particular lineage divergences in the tree of life. Cofactor biosynthetic networks are themselves modular, with multiple biosynthetic pathways in a family using closely related enzymes that enable structures characteristic of the cofactor class.

The quite sharply defined roles of many modules enable us to understand strong evolutionary convergences that have occurred within fundamental biochemistry, and in some cases we can relate the functioning of an entire class of substrate or control molecules to specific chemical properties of elements or small chemical groups. Several important module boundaries are aligned at the same points in their substrate networks and their control layers. This suggests to us that lower-level substrate-reaction networks introduced constraints on the accessible or robust forms of catalysis and aggregation that it was later possible to build up over them. From repeated motifs within the substructure of modules, and from patterns of re-use or convergence, we may identify chemical constraints on major transitions in metabolic evolution, and we may separate the early functions of promiscuous catalysts as enablers of chemistry, from later restrictions of reactants as catalysts were made more specific. The remarkable fact that such low-level chemical distinctions (in elements, reactions, or small-molecule networks) should have created constraints on innovation well into the Darwinian era of modern cells suggests these as relevant constraints also in the pre-cellular era.

### C. Manuscript outline

Our main message is twofold: 1) that the structure of biosynthetic networks and their observed variation, even though the networks are elaborate, has a compact representation in terms of a small collection of rules for composition, and 2) that the same rules we abstract from composition have a natural interpretation as constraints on evolutionary dynamics, which as a generating process has produced the observed variants. We intend the expression “logic of metabolism” to refer to the collection of architectural motifs and functions that have apparently been necessary for persistence of the biosphere, that have led to modularity in the physics and chemistry of life, and that have determined its major evolutionary contingencies and convergences.

After a short description of the important global features of metabolism in Sec. II, we will construct these at ascending levels in the hierarchy, beginning in Sec. III

with the networks of core carbon fixation and the lowest levels of intermediary metabolism. We will then, in Sec. IV, consider cofactors as the intermediate level of structure and the first level of explicit control in biochemistry, illustrating how key cofactor classes govern the fixation and transfer of elementary carbon units, and introduce control over reductants and redox state. Both in the metabolic substrate and in the cofactor domain, it will be possible to suggest a specific historical order for many major innovations. For the substrate network this will capture conditional dependencies in the innovation of carbon fixation strategies. For cofactors it will allow us to approximately place the emergence of specific cofactor functionalities within the expansion of metabolic networks from inorganic inputs.

In Sec. V we consider the processes by which innovation occurs, specifically interplay of the introduction of general reaction mechanisms versus selectivity over substrates. The modular substructure and evolutionary sequence of many of our reconstructed innovations favors an early role for non-specific catalysts, with substrate selectivity appearing later. Finally in Sec. VI we list candidates for the major organizing constraints on integration of metabolism within cells. These include the role of compartments in linking energy systems, as well as the coupling of physiological and genetic individuality, which permit species differentiation, and complementary specialization within ecological assemblies.

## II. AN OVERVIEW OF THE ARCHITECTURE OF METABOLISM

### A. Anabolism and catabolism in individuals and ecosystems

Metabolic networks within organisms are commonly characterized [42, 43] as having three classes of pathways: 1) catabolic pathways that break down organic food to provide chemical “building blocks” or energy; 2) core pathways through which nearly all small metabolites pass during primary synthesis or ultimate breakdown, and 3) anabolic pathways that build up all complex chemicals from components originating in the core. This qualitative characterization (which may be complicated by salvage pathways and other cross-linkages) is supported by a strong statistical observation that most minimal pathways connecting pairs of metabolites consist of a catabolic and an anabolic segment connected through the core [44]. Thus, relatively speaking, the catabolic and anabolic pathways are less densely crosslinked than pathways within the core, from which they radiate. Catabolic pathways in a cell may be fed through physiological or trophic links to other cells or organisms, or they may break down food produced previously by the same cell and then stored. Fig. 2 illustrates schematically the relation of the three classes. Both catabolic and anabolic pathways may be large and somewhat diversified; the

core itself constitutes no more than a few hundred small metabolites [39, 40], most of which have functions that are universal throughout the biosphere.

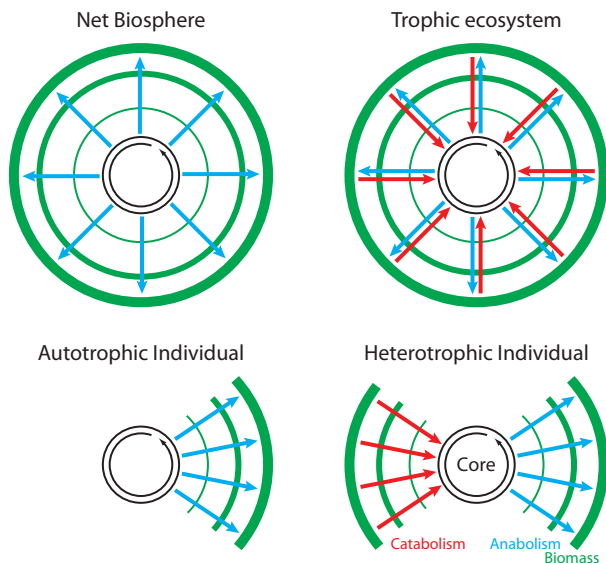


FIG. 2: Global structure of metabolism. Anabolic pathways (blue) build biomass and catabolic pathways (red), which may be their direct reverses, break it down. Carbon enters the biosphere through the core (black), which is the starting point of anabolism and also the endpoint of respiration. Because the biosphere as a whole is autotrophic, anabolism is functionally prior to catabolism. Both single organisms and assemblies of autotrophs can possess metabolic charts consisting only of anabolic pathways that fan outward from the core (blue and green). By partitioning pathway directions between anabolic and catabolic (joined at the core), organisms can take on the familiar “bowtie” architecture of derived metabolism (red with blue). Their assembly into trophic ecosystems (blue and red radial graph) then both builds and degrades organic compounds actively, cycling carbon between environmental  $\text{CO}_2$  and biomass (green). In these graphs, concentric (green) shells reflect sequential steps in biosynthesis leading to a hierarchy of increasing molecular complexity.

Whole-organism metabolisms are conventionally divided into two classes – autotrophs and heterotrophs – according to the ways they combine anabolic and catabolic pathways [5]. Autotrophs synthesize all required metabolites from inorganic precursors, and can function without catabolism, using only the core and anabolic pathways radiating from it.<sup>6</sup> Heterotrophs, in contrast, are organisms that must obtain organic inputs from their environments because they lack essential biosynthetic pathways. As a result of this difference, the two classes of metabolism have fundamentally different ecological roles.

<sup>6</sup> Establishing this completeness can prove challenging, however [45].

Autotrophs form the lowest trophic level in the biosphere, fixing  $\text{CO}_2$  into organic matter, while heterotrophs form all subsequent levels, determining the structure of flows of organic compounds in trophic webs [46], and actively cycling carbon from biomass back to environmental  $\text{CO}_2$ . While all biological free energy passes at some stage through redox couples, autotrophs capture a part of this energy by transferring electrons from high energy reductants to  $\text{CO}_2$  [7]. Heterotrophs may exploit incomplete use of this free energy through internal redox reactions (fermentation), or they may re-oxidize organic matter back to  $\text{CO}_2$  (respiration).

The role of catabolism in most organisms is closely tied to their ecological role as heterotrophs. Heterotrophy provides enormous opportunity for metabolic diversification [34], in the evolution of catabolic pathways and the partitioning of essential anabolic reactions among the constituent species within ecosystems. However, the study of metabolism restricted to particular heterotrophic organisms<sup>7</sup> can obscure much of its universality: heterotrophs may differ widely, but the aggregate anabolic networks that sustain them at the level of ecosystems are largely invariant. Autotrophs show that much of this diversity is not essential to life, allowing us to conceptually separate the requirements for biosynthesis from complexities that originate in processes of individual specialization and ecological assembly [47].

The motif of three-stage pathways – catabolic, core, anabolic – between typical pairs of metabolites, a motif obtained through the study of heterotrophs, has been abstracted into a paradigm of “bowtie” architecture for metabolism [42–44].<sup>8</sup> However, in combining universal elements of metabolic dependency with widely variable physiological or ecological specializations, the “bowtie” can be misleading. The core and anabolism are essential (and we argue more ancestral), and the reduction in cross-linking with distance from the core may be seen to reflect an entirely outgoing radial “fan” of anabolism. Biomass is organized in a sequence of concentric shells spanned by the radial pathways, which count the number and complexity of biosynthetic steps [40]. Organisms exist which can function without catabolism, but only the most derived parasites lack anabolism. Moreover, many of the common catabolic pathways are (approximate or

<sup>7</sup> Almost all model organisms have been heterotrophs, because these are accessible and are usually connected to humans as symbionts, pathogens, or cultivars. *E. coli* (in which operons were discovered) is a phenotypically and trophically very plastic organism due to its complex lifecycle. All multicellular organisms are heterotrophs, including plants, since these fix carbon but rely on soil symbionts to fix nitrogen. The only known autotrophic organisms are bacteria and archaea, and no autotroph is currently well-developed as a model organism.

<sup>8</sup> The paradigm of the metabolic bowtie is also in part a borrowing from a conventional paradigm in engineering [42], motivated by applications to human physiology and medicine (John Doyle, pers. comm.).

exact) reversals of widespread anabolic pathways,<sup>9</sup> and are explained as consequences of ecological change. Finer diversifications arise as adaptations to specific ecological or geochemical environments. Therefore, in this review we will emphasize core pathways and a subset of anabolic pathways, as they contribute to the universal aspects of autotrophic networks.

The conceptual difference and asymmetry between autotrophy and heterotrophy becomes clearer when we examine the metabolic structure of ecosystems at increasing scales of aggregation. Entire ecosystems, to the extent that they are approximately closed, function chemically as autotrophs. The biosphere as a whole is not only approximately, but fully autotrophic, as today it does not depend significantly on extraterrestrially, atmospherically, or geologically produced organics. This observation still admits two possibilities for the emergence of aggregate metabolism: Either the biosphere has been autotrophic since its inception, or it was originally heterotrophic and later switched to using CO<sub>2</sub> as its sole carbon source.

The Oparin-Haldane conjecture [49, 50] has motivated some consideration of a catabolic origin of life, but we can find no close empirical contact of this conjecture with features of extant life. Therefore we will assume the primacy of the core and anabolic pathways, and will consider the problem of emergence and early evolution of fully autotrophic systems. As long as we do not conflate the chemical condition of autotrophy (complete anabolism) with assumptions about individuality (whether complete anabolisms are contained within the regulatory control of individual organisms) [47], and as long as we recognize the ecosystem as potentially the correct level of aggregation to define autotrophy, we need not assume that the first life consisted of autotrophic individual cells. Our interpretation extends equally to populations of organisms that were physiologically as well as genetically incomplete and functioned cooperatively [51–54].

Once organism-level and species-level organization has been put aside as a separate question, the chemical distinction between heterotrophy and autotrophy is a distinction between metabolic partial-systems with unknown and highly variable boundary conditions, versus whole-systems required to subsist on CO<sub>2</sub> and reductant. If we wish to understand the structure of the biosphere and to interpret the sequence of innovations in core carbon fixation, the added constraints of autotrophy provide a framework to do this. Finally, identifying the chemical nature of life with autotrophic metabolism, rather than modeling it on species heterotrophy as in the Oparin-Haldane conjecture, is compatible with a hypothesis of continuity in the emergence of life [47]. Rather than re-inventing metabolism as a palimpsest over earlier abi-

otic sources [55], we suppose that organic life subsumed and controlled organosynthetic process of geochemical origin [27, 56].

## B. Network topology, self-amplification, and levels of structure

Understanding either the emergence of life, or the robust persistence of the biosphere, requires understanding life’s capacity for exponential growth. Exponential growth results from proportional self-amplification of metabolic and other networks that have an “autocatalytic” topology [57–61] (see Fig. 3). Network autocatalysis is a term used to describe a topological (stoichiometric) property of the substrate network of chemical reactions. In a *catalytic* network, one or more of the network intermediates is needed as a substrate to enable the pathway to connect to its inputs or to convert them to outputs, but the catalytic species is regenerated by the stage at which the pathway completes. Network-catalytic pathways must therefore incorporate feedback and comprise one or more loops with regard to the internally produced molecules. An *autocatalytic* network is a catalytic network augmented by further reactions that convert outputs to additional copies of the network catalyst, rendering the pathway self-amplifying.<sup>10</sup>

Network autocatalysis is necessary to maintain dynamical ordered states, by re-concentrating inputs into a finite number of intermediates, against the disordering effects of thermodynamic decay and continual external perturbation. Therefore all observed persistent material flows in the biosphere can only be products of autocatalytic networks, though they may require hard-to-recognize feedbacks ranging from the level of cell metabolism to trophic ecology for full regeneration. This *ex post* observation does not, however, explain why self-amplification was possible in abiotic chemistry to give rise to a biosphere. In addition to topologies enabling feedback, the latter would have required that intermediates in the network be produced at rates higher than those at which they were removed.

The significant observations about autocatalysis in the extant biosphere, which may also contain information

<sup>9</sup> An example is glycolysis, which is the reverse of gluconeogenesis [48].

<sup>10</sup> *Molecular autocatalysis* – the property that intermediates in a pathway serve as conventional molecular catalysts for other reactions in the pathway – may be understood as a restricted form of network autocatalysis in which the reaction to which some species is an essential input is the same reaction that regenerates that species. Some chemists prefer to use the term “network autoamplification” for the general case, restricting “autocatalysis” to apply only when species are traditionally-defined molecular catalysts. We will use “autocatalysis” for the general case, to reflect the property of stoichiometry that a pathway regenerates essential inputs. For us the distinction between autocatalysis at the single molecule versus more general network level mainly effects the kinetics and regulation of pathways.



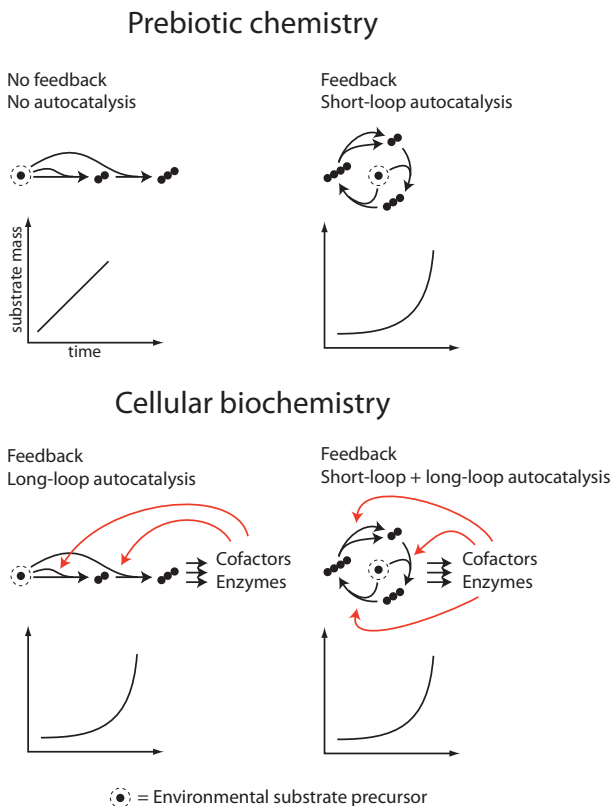


FIG. 3: Upper limit growth rate curves for chemical reaction networks of different classes. To highlight the role of network topologies, the chemistry is simplified with only C-C bond forming and cleaving reactions shown. Each of the growth curves qualitatively represents the upper limits for mass accumulation within the participating substrates of the pathways. When fully integrated within modern cellular biochemistry, both linear and cyclic pathways are network autocatalytic and capable of exponential growth. This form of network autocatalysis, however, derives from feedback provided by cofactors and enzymes, both of which have elaborate synthesis pathways, and is thus classified as “long-loop” autocatalysis. In an abiotic world in which reaction-level catalysis is limited to external sources, only cyclic pathways with feedback topologies at the substrate level – correspondingly classified as “short-loop” autocatalysis – are capable of exponential growth, while linear pathways are limited to linear growth. We contend that an early presence of short-loop autocatalysis is important because it provides a mechanism to concentrate mass flux within abiotic chemical networks, preventing excessive dilution with increasing size and complexity of organic molecules, and in turn giving easier and more robust access to subsequent long-loop feedback closures.

about its emergence, concern the complexity, number, and particular form of levels in which autocatalytic feedback can be found. Where the hierarchical modules of metabolic structure or function follow the boundaries required for feedback closure of different autocatalytic sub-networks, it may be possible to order the appearance of those sub-networks in time, and to infer

the geochemical supports they required for stability and self-amplification, before those supports were attained through integration into cellular biochemistry.

We wish, in these characterizations, to recognize what we might call “conditional” as well as strict autocatalysis. In extant organisms, where (essentially) all reactions are catalyzed by macromolecules, and most cofactors (reductants, nucleoside-triphosphates, coenzymes) are recharged by cellular processes, strict autocatalysis of any network is only satisfied in the context of the full complement of integrated cellular processes. If, however, inputs provided by cofactors, macromolecules and energy systems in modern cells could have been provided externally in earlier stages of life, for instance by minerals or geochemical processes, then identifying networks in extant biochemistry that, although simple, *would be* autocatalytic if given these supports, may give information about intermediate stages of emergence (see Fig. 3). The strong modularity of extant metabolism and its congruence with such conditionally autocatalytic topologies suggests that a separation into layers corresponding to stages of emergence may be sensible. In addition to reconstructing historical stages, the mechanisms leading to autocatalysis in different sub-systems may suggest important geochemical contexts or sources of robustness still exploited in modern metabolism.

### C. Network-autocatalysis in carbon-fixation pathways

At the chemically simplest level of description – that of the small-molecule metabolic substrates and their reaction-network topologies – carbon fixation pathways form two classes. Five of the the six known pathways are autocatalytic loops, while one is a linear reaction sequence.<sup>11</sup> The loop pathways condense  $\text{CO}_2$  or bicarbonate onto their substrate molecules, lengthening them. Each condensation is followed by a reduction, making the average oxidation state of carbon in the pathway substrate lower than that of the input  $\text{CO}_2$ , and resulting in a negative net free energy of formation in a reducing environment [7].<sup>12</sup> Each fixation loop contains one reaction where the maximal-length substrate is cleaved to produce two intermediates earlier in the same pathway, resulting in self-amplification of the pathway flux. As long as pathway intermediates are replenished faster

<sup>11</sup> All uses of autocatalysis in this section refer to *conditional* autocatalysis, taking as external support the same level of cofactor or enzymatic complexity. Such external factors being equal, the small-molecule substrate pathways of the loops display an additional form of autocatalysis not present in the linear pathway.

<sup>12</sup> Reducing power may originate in the geochemical environment, but in modern cells electrons are transferred endergonically to more powerful reductants such as NADH, NADPH,  $\text{FADH}_2$ , or reduced ferredoxin.

than they are drained by parasitic or anabolic side reactions, the loop current remains above the autocatalytic threshold. However, the threshold is fragile, as pathway kinetics provide no inherent barrier against flux's falling below threshold and subsequently collapsing.<sup>13</sup>

At the level of network topology, the linear *Wood-Ljungdahl* (WL) fixation pathway [62–64] is strikingly unlike the five loop pathways. Instead of covalently binding CO<sub>2</sub> onto pathway substrates, which then serve as platforms for reduction, the WL reactions directly reduce one-carbon (C<sub>1</sub>) groups, and then distribute the partly- or fully-reduced intermediates to other anabolic pathways where they are incorporated into metabolites. The linear sequence of reductions has no feedback, and the C<sub>1</sub> groups at intermediate oxidation states do not increase in complexity. Instead, these reductions (leading to intermediate C<sub>1</sub> states that would be unstable in solution) are carried out on evolutionarily refined folate cofactors [65]. The topology of the WL pathway becomes self-amplifying only if the larger and more complex biosynthetic network for these cofactors is considered together with that of the C<sub>1</sub> substrate. We will characterize this distinction between the loop-fixation pathways and WL as a distinction between *short-loop* and *long-loop* autocatalysis (see Fig. 3).<sup>14</sup> Short loops contain only the small-molecule substrates; long loops incorporate the biosynthetic networks for cofactors as well.

The network catalysts that could be said to “select” the short-loop pathways are the reaction intermediates themselves. The key metabolites that have the corresponding selection role for WL are the folate cofactors produced in a secondary biosynthetic network. Short-loop and long-loop pathways are therefore distinguished both by the number of reactions that must be maintained and regulated, and by the fact that WL spans substrates and cofactors, which we will argue in Sec. IV are naturally interpreted as qualitatively distinct layers within biochemistry.

The appearance of different features suggesting simplicity or primordial robustness, in different fixation pathways, together with aspects of their phylogenetic distribution, have led to diverse proposals about the order of their emergence [66, 67]. WL is the only carbon-fixation pathway found in both bacteria and archaea, and its reactions have been shown to have abiotic mineral analogues [66, 68, 69], suggesting a prebiotic origin. Yet WL is not self-amplifying and so lacks the capacity for chemical “competitive exclusion” (equivalent to the capacity for exponential growth). The cofactors that make

it self-amplifying are complex, and the simple pathway structure of C<sub>1</sub> reduction does not suggest what would have supported their formation.

In contrast, autocatalysis within the small-molecule substrate networks of the loop pathways suggests the inherent capacity for self-amplification, exponential growth, and chemical competitive exclusion. This is an appealing explanation [7] for the role, particularly of the intermediates in the reductive citric acid cycle [6, 70] (discussed in Sec. III) as precursors of biomass. Arcs within this pathway have also been reproduced experimentally in mineral environments [71], though a self-amplifying system has not yet been demonstrated. However, self-amplification requires complete loops, and even the most compelling candidate for a primordial form (reductive citric acid cycling) is found only in a subset of bacterial clades.

We argue in the next section that a joint fixation pathway incorporating both WL and citric acid cycling resolves many of these ambiguities in a way that no modern fixation pathway can.<sup>15</sup> As a phylogenetic root, it defines a template from which the fixation pathways in all modern clades could have diverged, and as a candidate for a primordial metabolic network, it provides both chemical selection of biomass precursors by short-loop autocatalysis, and a form of protection against the fragility of the autocatalytic threshold. We will first describe the biochemistry and phylogenetics of carbon-fixation pathways in the current biosphere, and then show how their patterns of modularity and chemical redundancy provide a framework for historical reconstruction.

### III. CORE CARBON METABOLISM

Currently six carbon fixation pathways are known [72, 73]. While they are distinct as complete pathways, they have significant overlaps at the level of individual reactions, and even greater redundancy in local-group chemistry. They are also, as shown in Fig. 5 (below), tightly integrated with the main pathways of core carbon metabolism, including lipid synthesis, gluconeogenesis, and pentose-phosphate synthesis.

An extensive analysis of their chemistry under physiologically relevant conditions has shown that individual fixation pathways contain two groups of thermodynamic bottlenecks: carboxylation reactions, and carboxyl reduction reactions [74]. In isolation these reactions generally require ATP hydrolysis to proceed, and how pathways deal with (or avoid) these costs has been

<sup>13</sup> The autocatalytic threshold and dynamics of growth, saturation, or collapse are considered in Sec. IIID, and shown in Fig. 12.

<sup>14</sup> In the network context of long-loop, WL fixation mechanism, the folate cofactors have an intermediate role between network catalysts and molecular catalysts, as they are passive carriers, but form stable molecular intermediates rather than mere complexes as are formed by enzymes with their substrates.

<sup>15</sup> A proposal for WL fixation followed by citric-acid cycling is made in the text of Ref. [69], though the primordial networks proposed in that paper are forms of acetogenesis, and do not emphasize self-amplification and short-loop autocatalysis as essential early requirements.



concluded to form an important constraint their internal structure [74]. We will further show how the elaborate and complex catalytic mechanisms associated with these reactions form essential *evolutionary* constraints on metabolism.

We will first describe the biochemical and phylogenetic details of the individual pathways, and then diagram their patterns of redundancy, first at the level of modular reaction sequences, and then in local-group chemistry. Finally we will use this decomposition together with evidence from gene distributions to propose their historical relation and identify constraints that could have spanned the Darwinian and pre-cellular eras.

## A. Carbon fixation pathways

### 1. Overview of pathway chemistries, phylogeny and environmental context

**Wood-Ljungdahl:** The Wood-Ljungdahl (WL) pathway [62–64, 66] consists of a sequence of five reactions that directly reduce one  $\text{CO}_2$  to a methyl group, a parallel reaction reducing  $\text{CO}_2$  to  $\text{CO}$ , and a final reaction combining the methyl and  $\text{CO}$  groups with each other and with a molecule of Coenzyme-A (CoA) to form the thioester acetyl-CoA. The reactions are shown below in Fig. 4, and discussed in detail in Sec. IV. The five steps reducing  $\text{CO}_2$  to  $-\text{CH}_3$  make up the core pathway of folate (vitamin  $\text{B}_9$ ) chemistry and its archaeal analog, which we consider at length in Sec. IIIB. The reduction to  $\text{CO}$ , and the synthesis of acetyl-CoA, are performed by the bi-functional CO-Dehydrogenase/Acetyl-CoA Synthase (CODH/ACS), a highly conserved enzyme complex with  $\text{Ni}[\text{Fe}_4\text{S}_5]$  and  $\text{Ni-Ni}[\text{Fe}_4\text{S}_4]$  centers [75–78]. Methyl-transfer from pterins to the ACS active site is performed by a corrinoid iron-sulfur protein (CFESP) in which the cobalt-tetrapyrrole cofactor cobalamin (vitamin  $\text{B}_{12}$ ) is part of the active site [79, 80].

Phylogenetically, WL is a widely distributed pathway, found in a variety of both bacteria and archaea, including acetogens, methanogens, sulfate reducers, and possibly anaerobic ammonium oxidizers [72]. The full pathway is found only in strict anaerobes, because the CODH/ACS is one of the most oxygen-sensitive enzymes known [81, 82]<sup>16</sup>. However, as we have argued in Ref. [41], the folate-mediated reactions form a partly-independent sub-module. This module combines with the equally-distinctive CODH/ACS enzyme to form the complete

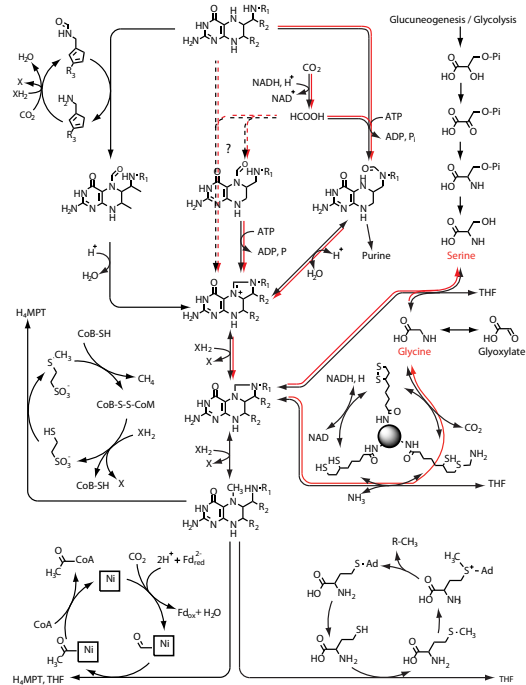


FIG. 4: The reactions in the Wood-Ljungdahl pathway of direct  $\text{C}_1$ -reduction. The main sequence on pterins is shown, with five outputs for formyl, methylene, or methyl groups. The semi-independent sub-module often used to directly synthesize glycine and serine from  $\text{CO}_2$ , even when acetyl-CoA synthesis is absent, is highlighted in red. Alternative pathways to glycine and serine, from 3-phosphoglycerate in gluconeogenesis/glycolysis and glyoxylate, are shown in the upper right quadrant. Finally, the dashed arrows represent a suggested alternative form of formate uptake based on binding at  $\text{N}^5$  rather than  $\text{N}^{10}$  of folate before cyclization to methenyl-THF [41].

WL pathway, but can serve independently as partial carbon-fixation pathways even in the absence of the final step to acetyl-CoA (see Fig. 4). In this role it is found almost universally among deep bacterial clades.

All carbon fixation pathways in extant organisms employ some essential and apparently unique enzymes and most also rely in essential ways on certain cofactors.<sup>17</sup> Among these, however, the function provided by pterin cofactors<sup>18</sup> in WL is arguably the most complex, extending beyond mere reduction. Pterins mediate capture of

<sup>16</sup> Recent results (S. Ragsdale, pers. comm.) suggest that the CODH/ACS is also sensitive to sulfides and perhaps other oxidants. We will impute this oxidant sensitivity as the reason the CODH/ACS was lost in the ancestral environments of some clades in Sec. IIID2. For later branches the oxidant may have been molecular oxygen, but  $\text{O}_2$  is not a plausible toxin at the time of the LUCA or earliest phylogenetic separations.

<sup>17</sup> For example, the 3-hydroxypropionate pathway relies on biotin for reactions shared with (or homologous to) those in fatty acid synthesis. The reductive citric-acid cycle relies on reduced ferredoxin [83], a simple multi-domain  $[\text{Fe}_4\text{S}_4]$  enzyme and on thiamine, in its reductive carbonyl-insertion reaction [84], and also on biotin for its  $\beta$ -carboxylation steps [85, 86].

<sup>18</sup> *Pterin* is a name referring to the class of cofactors including folates and the methanopterins, which are both derived from a neopterin precursor.

formate, reduction of carbon bound to one or two nitrogen atoms, and transfer of formyl, methylene, or methyl groups.<sup>19</sup> In this sense the simple network topology of direct  $C_1$  reduction seems to require a more elaborate dependence on cofactors than is seen in other pathways.

**Reductive citric-acid cycle:** The reductive citric-acid (reductive Tricarboxylic Acid, or rTCA) cycle [70, 87] is the reverse of the oxidative Krebs cycle, which was by all evidence derived from it [7, 33, 88] during the rise of oxygen. It is a sequence of eleven intermediates and eleven reactions, highlighted in Fig. 5, which reduce two molecules of  $CO_2$ , and combine these through a substrate-level phosphorylation with CoA, to form one molecule of acetyl-CoA. In the cycle, one molecule of oxaloacetate grows by condensation with two  $CO_2$  and is reduced and activated with CoA. The result, citryl-CoA, undergoes a retro-aldol cleavage to regenerate oxaloacetate and acetyl-CoA.<sup>20</sup> A second arc of reactions sometimes termed *anaplerotic* [5] condenses two further  $CO_2$  with acetyl-CoA to produce a second molecule of oxaloacetate, completing the network-autocatalytic topology and making the cycle self-amplifying. The distinctive reaction in the rTCA pathway is a carbonyl insertion at a thioester (acetyl-CoA or succinyl-CoA), performed by a family of conserved ferredoxin-dependent oxidoreductases which are triple- $Fe_4S_4$ -cluster proteins [84]. The cycle is found in many anaerobic and microaerophilic bacterial lineages, including aquificales, chlorobi, and  $\delta$ - and  $\epsilon$ -proteobacteria.

Enzymes from reductive TCA reactions are very widely distributed among bacteria, where they support fermentative pathways that break cycling and use intermediates such as succinate as terminal electron acceptors [91]. The co-presence of variant enzymes associated with reductive and oxidative cycling [92–94] may provide detailed evidence about the reversal of core metabolism under the rise of oxygen.

**Dicarboxylate/4-hydroxybutyrate cycle:** The dicarboxylate/4-hydroxybutyrate (DC/4HB) cycle [66, 95], illustrated in Fig. 7 (below) is, like rTCA, a single-loop network-autocatalytic cycle, but has a simpler form of autocatalysis in which acetyl-CoA rather than oxaloacetate is the network catalyst. Only two  $CO_2$  molecules are attached in the course of the cycle to form acetoacetyl-CoA, which is then thioesterified at the second acetyl moiety and cleaved to directly regenerate two molecules of acetyl-CoA. An extra copy of the network catalyst is thus directly regenerated (with suitable CoA

activation) without the need for anaplerotic reactions. The cycle has so far been found only in anaerobic crenarchaeota, but within this group it is believed to be widely distributed phylogenetically [66, 95]. The first five reactions in the cycle (from acetyl-CoA to succinyl-CoA) are identical to those of rTCA. The second arc of the cycle begins with reactions found also in 4-hydroxybutyrate and  $\gamma$ -aminobutyrate fermenters in the clostridia (a subgroup of Firmicutes within the bacteria), and terminates in the reverse of reactions in the isoprene biosynthesis pathway. The DC/4HB pathway thus uses the same ferredoxin-dependent carbonyl-insertion reaction used in rTCA (though only at acetyl-CoA), along with distinctive reactions associated with 4-hydroxybutyrate fermentation. In particular, the dehydration/isomerization sequence from 4-hydroxybutyryl-CoA to crotonyl-CoA is performed by a flavin-dependent protein containing an  $[Fe_4-S_4]$  cluster, and involves a ketyl-radical intermediate [96, 97].

**3-hydroxypropionate bicycle:** The 3-hydroxypropionate (3HP) bicycle [82], highlighted in Fig. 9, has the most complex network topology of the fixation pathways, using two linked cycles to regenerate its network catalysts and to fix carbon. The network catalysts in both loops are acetyl-CoA and the outlet for fixed carbon is pyruvate. The reactions in the cycle begin with the biotin-dependent carboxylation of Acetyl-CoA to form Malonyl-CoA, from the fatty-acid synthesis pathway, followed by a distinctive thioesterification [98] and a second, homologous carboxylation of propionyl-CoA (to methylmalyl-CoA) followed by isomerization to form succinyl-CoA. The first cycle then proceeds as the *oxidative* TCA arc, followed by retro-aldol reactions also found in the glyoxylate shunt (described below). A second cycle is initiated by an aldol condensation of propionyl-CoA with glyoxylate from the first cycle to yield  $\beta$ -methylmalyl-CoA, which follows a sequence of reduction and isomerization through an enoyl intermediate (mesaconate) similar to the second cycle of rTCA or the 4HB pathway. This complex pathway was discovered in the Chloroflexi and is believed to represent an adaptation to alkaline environments in which the  $CO_2/HCO_3^-$  (bicarbonate) equilibrium strongly favors bicarbonate. All carbon fixations proceed through activated biotin, thus avoiding the carbonyl insertion of the rTCA and DC/4HB pathways. The complexity of the bicycle network likely reflects the difficulty of replacing *both* carbonyl insertion reactions from an ancestral rTCA cycle while retaining autocatalysis, but it also suggests the diverse inventory of pathway segments available to draw from at the time of its emergence, which reflect an underlying chemical simplicity and redundancy, as we will show.

**3-hydroxypropionate/4-hydroxybutyrate cycle:** The 3-hydroxypropionate/4-hydroxybutyrate (3HP/4HB) cycle [99], shown in Fig. 7, is a single-loop pathway in which the first arc is the 3HP pathway, and the second arc is the 4HB pathway. Like DC/4HB,

<sup>19</sup> The distinctive role of cofactors continues with the dependence of the acetyl-CoA synthesis on cobalamin, a highly reduced tetrapyrrole capable of two-electron transfer [79].

<sup>20</sup> Here we separate the formation of citryl-CoA from its subsequent retro-aldol cleavage, as this is argued to be the original reaction sequence, and the one displaying the closest homology in the substrate-level phosphorylation with that of succinyl-CoA [89, 90].

3HP/4HB uses acetyl-CoA as network catalyst and fixes two  $\text{CO}_2$  to form acetoacetyl-CoA. The pathway is found in the Sulfolobales (crenarchaeota), where it combines the crenarchaeal 4HB pattern of autotrophic carbon fixation with the bicarbonate adaptation of the 3HP pathway. Like the 3HP bicycle, the 3HP/4HB pathway is thought to be an adaptation to alkalinity, but because the 4HB arc does not fix additional carbon, this adaptation resulted in a simpler pathway structure than the bicycle.

**Calvin-Benson-Bassham cycle:** The Calvin-Benson-Bassham (CBB) cycle [100, 101] is responsible for most of known carbon fixation in the biosphere. In the same way as WL adds only the distinctive CODH/ACS reaction to an otherwise very-widely-distributed folate pathway [41], the CBB cycle adds a single reaction to the otherwise-universal network of aldol reactions among sugar-phosphates that make up the gluconeogenic pathway to fructose 1,6-bisphosphate and the reductive pentose phosphate pathway to ribose and ribulose 1,5-bisphosphate.<sup>21</sup> The distinctive CBB reaction that extends reductive pentose-phosphate synthesis to a carbon fixation cycle is a carboxylation performed by the Ribulose 1,5-bisphosphate Carboxylase/Oxygenase (Ru-bisCO), together with cleavage of the original ribulose moiety to produce two molecules of 3-phosphoglycerate. The Calvin cycle resembles the 4HB pathways in regenerating two copies of the network catalyst directly, not requiring separate anaplerotic reactions for autocatalysis. In addition to carboxylation, RubisCO can react with oxygen in a process known as *photorespiration* [102–104] to produce 2-phosphoglycolate (2PG), a precursor to glyoxylate that is independent of rTCA-cycle reactions. The CBB cycle is widely distributed among cyanobacteria, in chloroplasts in plants, and in some secondary endosymbionts.

**The glyoxylate shunt:** Although it is not an autotrophic carbon-fixation pathway, the glyoxylate shunt (or glyoxylate bypass) is of interest because it shares intermediates and reactions with many of the above fixation pathways, and because it resembles a fixation pathway in certain topological features. The pathway is shown below in Fig. 9. All aldol reactions that can be performed starting from rTCA intermediates appear in this pathway, either as cleavages or as condensations. In addition to condensation of acetate and oxaloacetate to form citrate, these include cleavage of isocitrate to form

glyoxylate and succinate, and condensation of glyoxylate and acetate to form malate. The shunt is a weakly oxidative pathway (generating one  $\text{H}_2$ -equivalent from oxidizing succinate to fumarate), and is otherwise a network of internal redox reactions. It is therefore a very widely-used facultative pathway under conditions where carbon for biosynthesis, more than reductant, is limiting.

Two of the arcs of the shunt overlap with arcs in the oxidative Krebs cycle, but the entire pathway is a bicycle much like the 3HP-bicycle, sharing many of the same intermediates, but running in the opposite direction. Oxidative pathways such as the Krebs cycle are ordinarily catabolic, and hence not self-maintaining. The glyoxylate shunt may be regarded as a network-autocatalytic pathway for intake of acetate, using malate as the network catalyst and regenerating a second molecule of malate from two acetate molecules. This may be part of the reason that the shunt is up-regulated in the *deinococcus-thermus* family of bacteria in response to radiation exposure [105], providing additional robustness from network topology under conditions when metabolic control is compromised.

## 2. Thermodynamic constraints on pathway structure

The central energetic costs of carbon-fixation pathways are associated with carboxylation reactions in which  $\text{CO}_2$  molecules are added to the growing substrate, and the subsequent reactions in which the carboxyl group is reduced to a carbonyl [74]. In isolation these reactions require ATP hydrolysis, but these costs can be avoided in several ways. In some cases a thioester intermediate is used to effectively couple together a carboxyl reduction and a subsequent carboxylation, allowing the two reactions to be driven by a single ATP hydrolysis. An unfavorable (endergonic) reaction can also be coupled to a highly favorable (exergonic) reaction, allowing the reactions to proceed without ATP hydrolysis.

Individual pathways employ such couplings to varying degrees, resulting in a range of ATP costs associated with carbon fixation. At the low end, WL eliminates nearly all use of ATP through its unique pathway chemistry. Using folates to reduce one-carbon units derived from  $\text{CO}_2$  before incorporating them into growing substrates avoid the cost of carboxylation, saving an ATP, while the endergonic reduction of  $\text{CO}_2$  to CO is coupled to the subsequent exergonic synthesis of acetyl-CoA. Finally, the activated thioester bond of acetyl-CoA allows the subsequent carboxylation to pyruvate to also proceed without additional ATP. As a result WL requires on a single ATP, for the attachment (and activation) of formate on  $\text{THF}^{22}$ , in the synthesis of pyruvate from  $\text{CO}_2$  [66, 74]. Similarly,

<sup>21</sup> The universality of this network requires some qualification. We show a canonical version of the network in the figures below, and some variant on this network is present in every organism that synthesizes ribose. However, the  $(\text{CH}_2\text{O})^n$  stoichiometry of sugars, together with the wide diversity of possible aldol reactions among sugar-phosphates, make sugar re-arrangement a problem in the number theory of the small integers, with solutions that may depend sensitively on allowed inputs and outputs. Other pathways within the collection of attested pentose-phosphate networks are shown in Ref. [61].

<sup>22</sup> In methanogens this cost has been completely eliminated by modifying the structure of THF to that of  $\text{H}_4\text{MPT}$  [41]

rTCA has high energetic efficiency as a result of extensive reaction coupling, requiring only 2 ATP to synthesize pyruvate from CO<sub>2</sub> [66, 74]. Two ATP are saved by coupling carboxyl reductions to subsequent carboxylations using thioester intermediates, and an additional ATP is saved by coupling the carboxylation of  $\alpha$ -ketoglutarate to the subsequent carbonyl reduction leading to isocitrate.

At the high end of energetic cost of carbon-fixation are pathways that couple unfavorable reactions less effectively, or not at all, or even hydrolyze ATP for reactions other than carboxylation or carboxyl reduction. Both the DC/4HB pathway and the 3HP bicycle decouple one or more of the thioester-mediated carboxyl reduction + carboxylation sequences such as used in rTCA, and neither couple endergonic carboxylations to exergonic reductions. As a result DC/4HB requires 5 ATP and the 3HP bicycle 7 ATP to synthesize pyruvate from CO<sub>2</sub> [66, 74]. The 3HP/4HB pathway has the highest cost of any fixation pathway, with 9 ATP required to synthesize pyruvate from CO<sub>2</sub>. This is partly because it also decouples thioester-mediated carboxyl reduction + carboxylation sequences, and partly because pyruvate is synthesized by diverting and ultimately *decarboxylating*

<sup>23</sup> Here, by *stoichiometry* we refer to the mole-ratios of reactants and products for each reaction, with molecules represented by their CHO constituents, and attached phosphate or thioester groups omitted. Where phosphorylation or thioesterification mediates a net dehydration, we have represented the dehydration

succinyl-CoA [66, 73, 74]. Finally, CBB is also at the high end in terms of cost, requiring 7 ATP to synthesize pyruvate from CO<sub>2</sub>. Although this pathway avoids the cost of carboxylation reactions by coupling them to exergonic cleavage reactions, CBB is the only fixation pathway that invests ATP hydrolysis in chemistry other than carboxylations or carboxyl reductions, relatively increasing its cost [74].

### 3. Centrality and universality of the reactions in the citric-acid cycle, and the pillars of anabolism

The apparent diversity of six known fixation pathways is unified by the role of the citric-acid cycle reactions, and secondarily by that of gluconeogenesis and the pentose-phosphate pathways. Fig. 5 presents the C,H,O stoichiometry<sup>23</sup> for a network of reactions that includes all six known pathways. The network contains only 35 organic intermediates,<sup>24</sup> because many intermediates and reactions appear in multiple pathways.

directly in the figure.

<sup>24</sup> Hydroxymethyl-glutarate and butyrate are also shown, to indicate points of departure to isoprene and fatty acid synthesis, respectively.

In Fig. 5 the TCA cycle and the gluconeogenic pathway are highlighted. Beyond being mere points of departure for alternative fixation pathways and for diversifications in intermediary metabolism, they are invariants under diversification because they determine carbon flow among the universal precursors of biosynthesis.

Almost all anabolic pathways in extant organisms originate in one of five intermediates in the TCA cycle – acetate (as acetyl-CoA), pyruvate, oxaloacetate, succinate (or succinyl-CoA) or  $\alpha$ -ketoglutarate – which have been dubbed the “pillars of anabolism” [40]. Succinyl-CoA can serve as the precursor to pyrroles (metal-coordinating groups in many cofactors) – mainly in  $\alpha$ -proteobacteria and mitochondria – but these are more commonly made from  $\alpha$ -ketoglutarate *via* glutamate in what is known as the C5 pathway [106]. A phylogenetic analysis of these pathways confirms that the C5 pathway is the most plausible ancestral form [107]. Thus as few as four TCA intermediates provide the organic inputs to all anabolic pathways. Fig. 6 shows the major molecule classes associated with each intermediate. The only exceptions to this universality, which form a biosynthetic sequence, are glycine, serine, and a few compounds synthesized from them; this sequence can be initiated directly from CO<sub>2</sub> outside of the pillars (see Fig. 4), an observation that becomes key in reconstructing the evolutionary history of

carbon-fixation (see Sec. III C). The gluconeogenic pathway then forms a similarly unique bridge between the TCA intermediate pyruvate (in the activated form phosphoenolpyruvate) and the network of sugar-phosphate reactions known as the *pentose-phosphate pathway*.

Carbon-fixation pathways must reach all four (or five) of the universal anabolic starting compounds. They may do this either by producing them as pathway intermediates, or by means of secondary reactions converting pathway intermediates into the essential precursors. The degree to which a pathway passes through all essential biosynthetic precursors may suggest its antiquity. In metabolism-first theories of the origin of life [6], the limited set of compounds selected and made available in high concentration by proto-metabolism determined the opportunities for further biosynthesis, thus establishing themselves as the precursors of anabolism.

Among the five network-autocatalytic fixation pathways, the CBB pathway is unique in not passing through *any* universal anabolic precursors. When used as a fixation pathway, CBB reactions must thus be connected to the rest of anabolism through the reverse of several reactions in the gluconeogenic pathways connecting 3-phosphoglycerate (3PG) to pyruvate. Pyruvate is then connected to the remaining precursors through partial TCA sequences. Glyoxylate produced from 2-

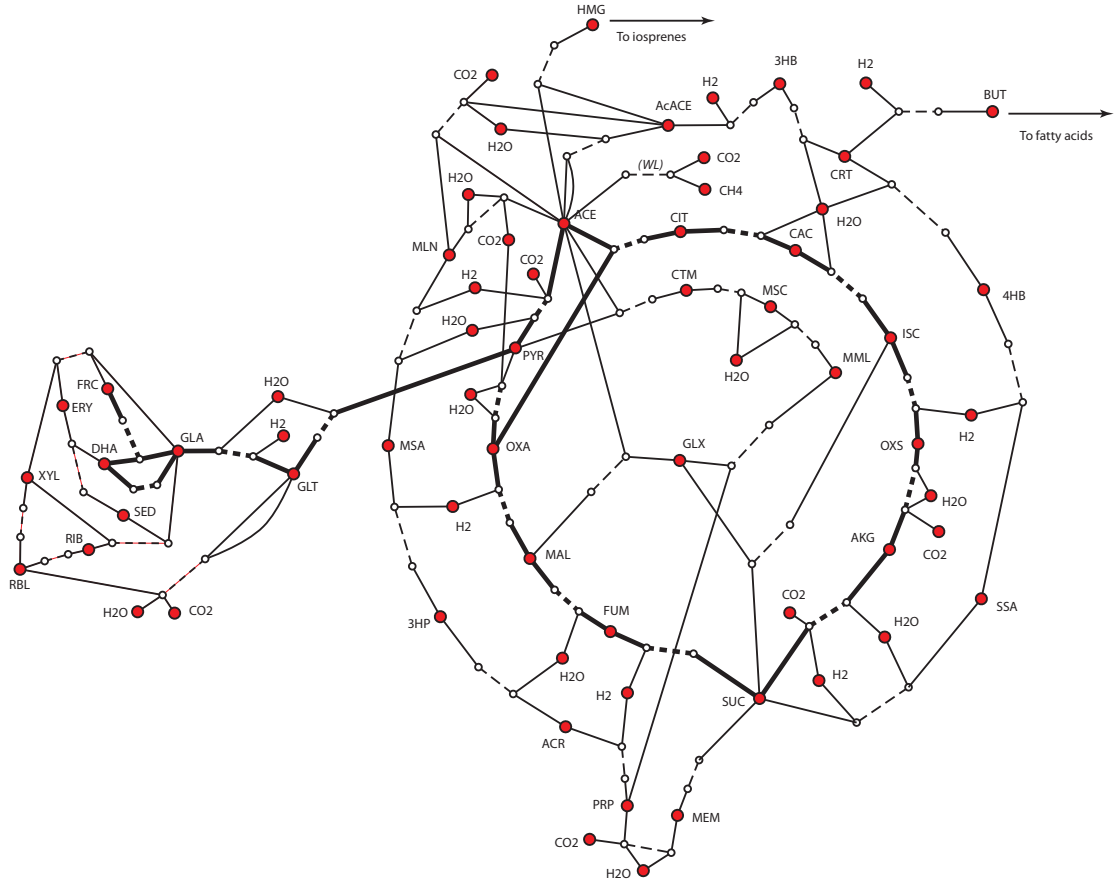


FIG. 5: The projection of the complete network for core carbon anabolism onto its CHO components. Phosphorylated intermediates and thioesters with Coenzyme-A are not shown explicitly. The bipartite graph notation used to show reaction stoichiometry is explained in App. A. Arcs of the reductive citric acid cycle and gluconeogenesis are bold, showing that these pathways pass through the universal biosynthetic precursors. The Wood-Ljungdahl (labeled *WL*) pathway, without its cofactors and reductants shown, is represented by the last reaction of the acetyl-CoA synthase, which is the inverse of a disproportionation. Abbreviations: acetate (ACE); pyruvate (PYR); oxaloacetate (OXA); malate (MAL); fumarate (FUM); succinate (SUC);  $\alpha$ -ketoglutarate (AKG); oxalosuccinate (OXS); isocitrate (ISC); cis-aconitate (CAC); citrate (CIT); malonate (MLN); malonate semialdehyde (MSA); 3-hydroxypropionate (3HP); acrylate (ACR); propionate (PRP); methylmalonate (MEM); succinate semialdehyde (SSA); 4-hydroxybutyrate (4HB); crotonate (CRT); 3-hydroxybutyrate (3HB); acetoacetate (AcACE); butyrate (BUT); hydroxymethyl-glutarate (HMG); glyoxylate (GLX); methyl-malate (MML); mesaconate (MSC); citramalate (CTM); glycerate (GLT); glyceraldehyde (GLA); dihydroxyacetone (DHA); fructose (FRC); erythrose (ERY); sedoheptulose (SED); xylulose (XYL); ribulose (RBL); ribose (RIB).

phosphoglycolate during photorespiration may alternatively be converted directly to glycine and serine (see Fig. 4).

Among the remaining loop-fixation pathways, only rTCA passes through all five anabolic pillars. Through its partial overlap with rTCA, DC/4HB passes through four, excluding  $\alpha$ -ketoglutarate. The 3HP-bicycle further bypasses oxaloacetate, while the 3HP/4HB loop and WL include only acetyl-CoA. All of the latter pathways require anaplerotic reactions in the form of incomplete (either oxidative or reductive) TCA arcs; when these combine (in various ways) with WL carbon fixation, they are known collectively as the *reductive acetyl-CoA pathways*.

The most parsimonious explanation for the universality of the TCA arcs as anaplerotic reactions is lock-in

by downstream anabolic pathways, to which metabolism was committed by the time carbon-fixation strategies diverged. This is a direct extension of the metabolism-first assumption that anabolic pathways themselves formed around proto-metabolic selection of the rTCA intermediates.<sup>25</sup> (A similar but later form of commitment has been argued to convert basal gene regulatory networks in metazoan development into *kernels*, which admit no variation and act as constraints on subsequent evolutionary dynamics [108, 109].) If lock-in provides the correct interpretation of TCA universality, then much of the bur-

<sup>25</sup> Harold Morowitz summarizes this assumption with the phrase *metabolism recapitulates biogenesis* [6].

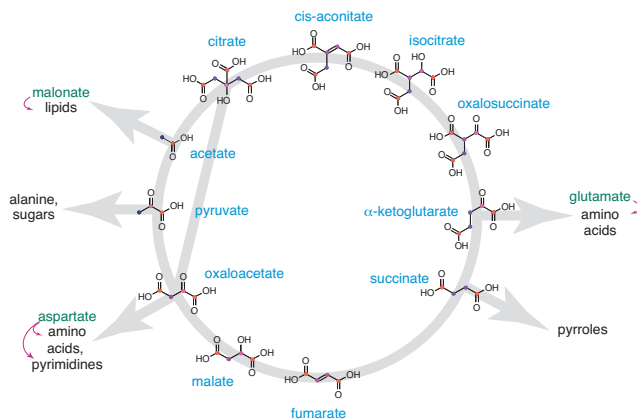


FIG. 6: The pillars of anabolism, showing lipids, sugars, amino acids, pyrimidines and purines, and tetrapyrroles from either succinate or AKG. Molecules with homologous local chemistry are at opposite positions on the circle. Oxidation states of internal carbon atoms are indicated by color (red = oxidized, blue = reduced).

den of accounting for the inventory of small metabolites is shifted away from Darwinian selection for function in a post-RNA world, and onto constraints of biosynthetic simplicity and network context. We show below that phylogenetic reconstruction is consistent with a selective role for rTCA cycling in the root metabolism of cellular life, though only as part of a larger network than the modern rTCA cycle.

### B. Modularity in the internal structure and mutual relationships of the known fixation pathways

Fig. 5 shows that the number of molecules and reactions required to include all carbon fixation pathways is much smaller than might have been expected from their nominal diversity, because many reactions are used in multiple pathways, and all pathways remain close to the universal precursors. We have already noted in the previous section that this re-use goes beyond the requirements of autocatalysis, to the anaplerotic role of rTCA arcs adapting variant fixation pathways to an invariant set of biosynthetic precursors.

The aggregate network also shows many kinds of structure: clusters, concentric rings, and ladders reflecting parallel sequences of the same inputs and outputs in different pathways. We will show in this section that these result from re-use of local-group chemistry in transformations of distinct molecules.

At the end of the section we will describe a third form of re-use not represented in the aggregate graph. The folate-mediated direct  $C_1$  reduction sequence of Wood-Ljungdahl, responsible for the methyl group in the WL disproportionation reaction in Fig. 5, is also found as a free-standing fixation pathway across the bacterial tree,

often as one component in a disconnected autotrophic network using one of the loop fixation pathways as its other component.

Because of such extensive redundancy, little innovation is required to explain the extant diversity of carbon fixation. All known carbon fixation strategies can be described as assemblies of a small number of strongly-defined *modules*, which govern not only the function of pathways, but also their evolution.

#### 1. Modularity in carbon fixation loops from re-use of pathway segments

Fig. 7 shows the sub-network from Fig. 5 containing the four loop-autotrophic carbon fixation pathways that pass through some or all universal precursors, together with reactions in the glyoxylate shunt. The four loop pathways are shown in four colors, with the organic pathway-intermediates (but not environmental precursors or reductants) highlighted.

The figure shows that these pathways re-use intermediates by combining entire *pathway segments*. The combinatorial assembly of these segments is possible because they all pass through acetate (as acetyl-CoA), succinate (usually as succinyl-CoA), and all except the second loop of the 3HP bicycle pass through both. Thus the conserved reactions among the autocatalytic loop carbon-fixation pathways are shared within strictly preserved sequences, which have key molecules as the boundaries at which segments may be combined.

#### 2. Homologous local-group chemistry across pathway segments

In addition to the re-use of complete reactions in pathway segments, variant carbon-fixation pathways have extensively re-used transformations at the level of local functional groups. The network of Fig. 7 is arranged in concentric rings, in which the arcs of the rTCA cycle align with the 3HP or 4HB pathways, or with the mesaconate arc of the 3HP bicycle. The “ladder” structure of inputs and outputs of reductant ( $H_2$ ) or water between these rings shows the similar stoichiometric progression in these alternative pathways. Fig. 8 decomposes the aggregate network into two pairs of short-molecule and long-molecule arcs, and the mesaconate arc, and shows the pathway intermediates in each arc. The figure makes clear that, both *within* the arcs of the loop pathways, and *between* alternate pathways, the type, sequence, and position of reactions is highly conserved. In particular, the reduction sequence from  $\alpha$ -ketones or semialdehydes, to alcohols, to isomerization through enoyl intermediates, is applied to the same bonds on the same carbon atoms from input acetyl moieties in rTCA, 3HP, and 4HB pathways, and to analogous functional groups in the bicycle. Finally, in the cleavage of both citryl-CoA



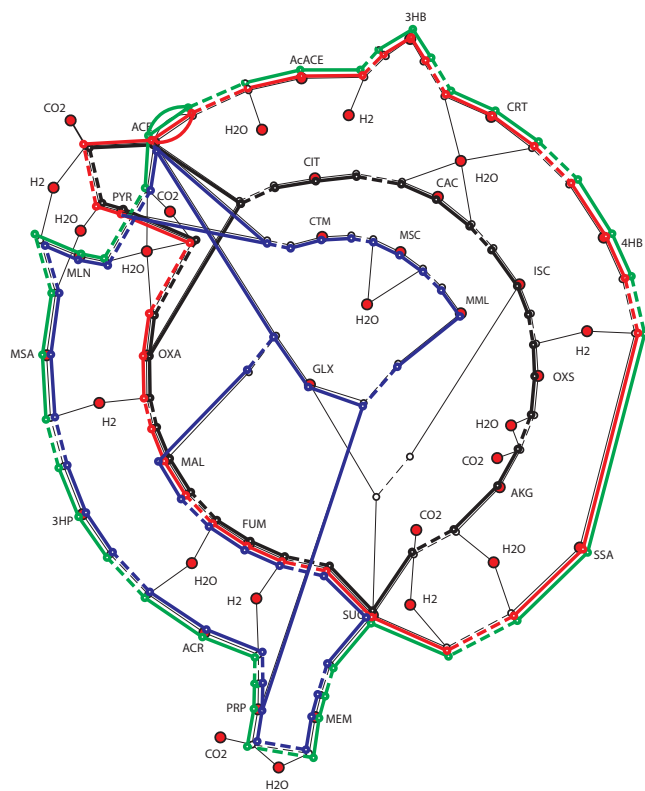


FIG. 7: The four loop carbon-fixation pathways that pass through some or all of the universal biosynthetic precursors, from the graph of Fig. 5. rTCA is black, DC/4HB is red, 3HP-bicycle is blue, and 3HP/4HB is green. The one aldol reaction from the glyoxylate shunt that is not part of the 3HP-bicycle is shown in fine lines. The module-boundary nature of acetate (ACE) and succinate (SUC) is shown by the intersection of multiple paths in these compounds. Radially aligned reactions are homologous in local-group chemistry; deviations from strict homology in different pathways appear as excursions from concentric circles.

and citramalyl-CoA, the bond that has been isomerized through the enoyl intermediate is the one cleaved to regenerate the network catalyst.

Even the distinctive step to crotonyl-CoA in the 4HB pathway creates an aconate-type intermediate, and the enzyme responsible has high homology to the acrolyl-CoA synthetase [110, 111], whose output (acrolyl-CoA) follows the standard pattern. Only the position of the double bond breaks the strict pattern in crotonyl-CoA, and the abstraction of the un-activated proton required to produce this bond requires the unique ketyl-radical intermediate [112]. From crotonyl-CoA, the sequence to 3-hydroxybutyrate is then followed by a surprising *oxidation* and re-hydration, resulting in a 5-step, redox-neutral, sequence. The net effect of this sequence is to shift the carbonyl group (of succinate semialdehyde, SSA) by one carbon (in acetoacetate, AcACE). Because the 4HB pathway takes in no new CO<sub>2</sub> molecules, this isomerization enables regeneration of the network cata-

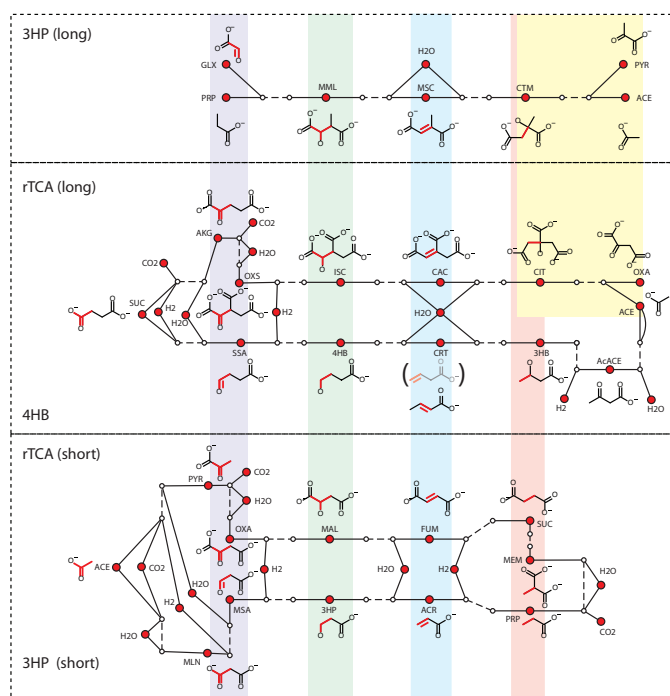


FIG. 8: Comparison of redundant reactions in the loop carbon fixation pathways. Pathways are divided into “long-molecule” (upper-ranks) and “short-molecule” (lower-ranks) segments; long-molecule segments occupy roughly the upper-right half-plane in Fig. 7, and abbreviations are as in Fig. 5. Molecule forms are shown next to the corresponding tags. Bonds drawn in red are the active acetyl or semialdehyde moieties in the respective segments. Vertical colored bars align homologous carbon states. The yellow block shows retro-aldol cleavages of citrate or citramalate. Two molecules are shown beneath the tag CRT (crotonate): the greyed-out molecule in parentheses would be the homologue to the other aconitase-type reactions; actual crotonate (full saturation) displaces the double bond by one carbon, requiring the abstraction of the  $\alpha$ -proton in 4-hydroxybutyrate via the ketyl-radical mechanism that is distinctive of this pathway.

lysts in the same way the reduction/aldol-cleavage sequence enables regeneration for rTCA or 3HP.

Duplication of reaction sequences in diverse fixation pathways results from retention of gene sets as organism clades diverged. Duplication of local-group chemistry in diverse reactions has resulted (at least in most cases) from retention of reaction mechanisms as enzyme families diverged. All enoyl intermediates are produced by a widely diversified family of aconitases [113], while biotin-dependent carboxylations are performed by homologous enzymes acting on pyruvate and  $\alpha$ -ketoglutarate [86],<sup>26</sup>

<sup>26</sup> Similar to the synthesis of citryl-CoA we separate here the carboxylation of  $\alpha$ -ketoglutarate from the subsequent reduction of oxalosuccinate to isocitrate – performed by a single enzyme in most organisms – because it is argued to be the ancestral

and substrate-level phosphorylation and thioesterification are similarly performed by homologous enzymes on citrate and succinate in rTCA [89, 90].<sup>27</sup> The wide coverage of a few reaction types may reflect their early establishment by promiscuous catalysts [116], followed by evolution toward increasing specificity as intermediary metabolic networks expanded and metabolites capable of participating in carbon fixation diversified.

A functional identification of modules that seeks to minimize influence from historical effects (such as lock-in) has been carried out by Noor *et al.* [117], and identifies similar module boundaries. Using as data the first three numbers of the EC classification of enzymes – which distinguish reaction types but coarse-grain over both substrate specificity and enzyme homology – they show that many pathways in core metabolism are the shortest routes possible between inputs and products. Where their analysis overlaps with the pathways shown here, many of their minimal sequences overlap with the modules in Fig. 7, as well as with others in gluconeogenesis which we do not consider here. Thus, returning to metabolism-first premises [6], it may be that historical retention of a few reaction types reflects facility of the substrate-level chemistry, and that this has placed time-independent constraints on evolution.

The functional-group homology shown in Fig. 8 allows us to separate stereotypical sequences of widely diversified reactions from key reactions that distinguish pathways. The stereotypical sequences lie downstream of reactions such as the ferredoxin-dependent carbonyl insertion (rTCA), or biotin-dependent carboxylation (3HP), which are associated with highly conserved enzymes or cofactors. The downstream reactions are also more “elementary”, in the sense that they are common and widely diversified in biochemistry, compared to the pathway-distinguishing reactions.

### 3. Association of the initiating reactions with transition-metal sulfide mineral stoichiometries and other distinctive metal-ligand complexes

The observation that alternative fixation pathways are not distinguished by their internal reaction sequences, but primarily by their initiating reactions, suggests that these reactions were the crucial bottlenecks in evolution, and perhaps reflect the limiting diversity of chemical mechanisms for carbon bond formation.<sup>28</sup> The distinctive use of metals in the (often highly conserved) en-

zymes and cofactors for these initiating reactions may further suggest a direct link between prebiotic mineral and metal-ligand chemistry [118], and constraints inferable from the long-term structure of later cellular evolution.

Several enzyme iron-sulfur centers have been recognized [119, 120] to use strained versions of the unit cells found in Fe-S minerals, particularly Mackinawite and Greigite. These are particular instances within a wider use of transition-metal-sulfide chemistry in core-metabolic enzymes.

Pyruvate:ferredoxin oxidoreductase (PFOR), which catalyzes the reversible carboxylation of acetyl-CoA to pyruvate, contains three  $[\text{Fe}_4\text{S}_4]$  clusters and a thiamin pyrophosphate (TPP) cofactor. The  $[\text{Fe}_4\text{S}_4]$  clusters and TPP combine to form an electron transfer pathway into the active site, and the TPP also mediates carboxyl transfer in the active site [84].

The bifunctional carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS) enzyme that catalyzes the final acetyl-CoA synthesis reaction in the WL pathway employs even more elaborate transition-metal chemistry. Like PFOR, this enzyme uses  $[\text{Fe}_4\text{S}_4]$  clusters for electron transfer, but its active sites contain additional, more unusual metal centers. The CODH active site contains an asymmetric  $[\text{Ni-Fe}_4\text{S}_5]$  cluster on which  $\text{CO}_2$  is reduced to CO [75], while the ACS active site contains a Ni-Ni- $[\text{Fe}_4\text{S}_4]$  cluster on which CO (from CODH) and a methyl group from folates are joined to form acetyl-CoA [76–78]. It was originally thought that a variant form of the ACS active site contains a Cu-Ni- $[\text{Fe}_4\text{S}_4]$  cluster [121, 122], but it was subsequently shown that the Cu-containing cluster represents an inactivated form of ACS [77]. Similarly, it has also been shown that the open form of the Ni-Ni ACS may switch to a closed, inactivated, form by exchanging one of the nickel atoms for a zinc atom [76]. Finally, methyl-group transfer from the methyl-pterin to the ACS active site is mediated by the corrinoid iron-sulfur protein (CFeSP) containing the cobalt-tetrapyrrole cofactor cobalamin [79, 80]. This transfer process involves the cycling between oxidation states of both the cobalt and one of the nickel atoms in the NiNi iron-sulfur cluster of ACS. In the first part of the transfer cobalt becomes oxidized from the Co(I) to the Co(III) state. The subsequent donation of the methyl-group to the ACS active site simultaneously reduces cobalt back to the Co(I) state and oxidizes the active nickel from the Ni(0) to the Ni(II) state. Finally, in the release of acetyl-CoA from the ACS the nickel is reduced back to the Ni(0) state, allowing the cycle to start over [80].

Perhaps not surprisingly, all these examples of metal-cluster enzymes concern catalysis not just of the formation of C-C bonds, but of the incorporation of the small gas-phase molecule  $\text{CO}_2$ . In general, enzymes involved in the processing of small gas-phase molecules (including  $\text{H}_2$  and  $\text{N}_2$ ) are among the most unique enzymes in biology

form [114, 115]

<sup>27</sup> However, the thioesterification of propionate is performed by distinct enzymes in bacteria and archaea, an observation that has been interpreted to suggest convergent evolution [82, 99].

<sup>28</sup> Mechanisms of organosynthesis in aqueous solution are especially limited by the instability of radical intermediates, which may be stabilized by association with metal centers.

– all but one of the known Nickel-containing enzymes belong to this group [123] – always containing highly complex metal centers in their active sites [124–129]. This indicates both the difficulty of controlling the catalysis of these reactions, and the importance of understanding their functions in the context of the origin of life [120].

#### 4. Complex network closures: diversity and opportunity created by aldol reactions

The network closures that retain carbon flux and enable autocatalysis in rTCA, DC/4HB, and 3HP/4HB pathways are all topologically rather simple, and are quite similar due to the homology among most of the pathway intermediates. Their module boundaries also are all defined by acetate and succinate, and at least in the case of acetate, were probably facilitated by its multiple pre-existing roles as the redox-drain of the rTCA cycle [33] and the starting point for both isoprenoid and fatty-acid lipid biosynthesis.

In contrast, the topology of the 3HP-bicycle appears complex, and perhaps an improbable solution to the problem of recycling all carbon flux through core pathways.<sup>29</sup> If we are to argue that the emergence or evolution of such network closures is facilitated by a form of modularity, it must exist at the level of reaction mechanisms that render their discovery less improbable. For the 3HP bicycle and the related glyoxylate shunt – and to a lesser degree also for rTCA – the mechanism of interest is the aldol reaction.

The aldol reaction is an internal oxidation-reduction reaction, which means that it exploits residual free energy from organosynthesis, and also that it can take place independently of external electron donors or acceptors. Many aldol reactions are also kinetically *facile*, occurring at appreciable rates without the aid of catalysts. We therefore expect that among compounds capable of participating in them, aldol reactions would have been common in the prebiotic world, providing opportunities for pathway generation. Since their diversity is difficult to suppress except by special mechanisms [130], we expect that potential aldol reactions among metabolites would either have become regulated (perhaps through phosphoryl occupation of hydroxyl groups) or else incorporated

into actively-used biochemical pathways.

Aldol reactions are important generators of diversity in organic chemistry, notorious for the very-complex network known as the *formose reaction* [131–134], initiated from formaldehyde and glycolaldehyde. Many aldol reactions are possible for sugars, and the reductive pentose phosphate pathway is indeed a network of selected aldol condensations and cleavages among sugar-phosphates [61].

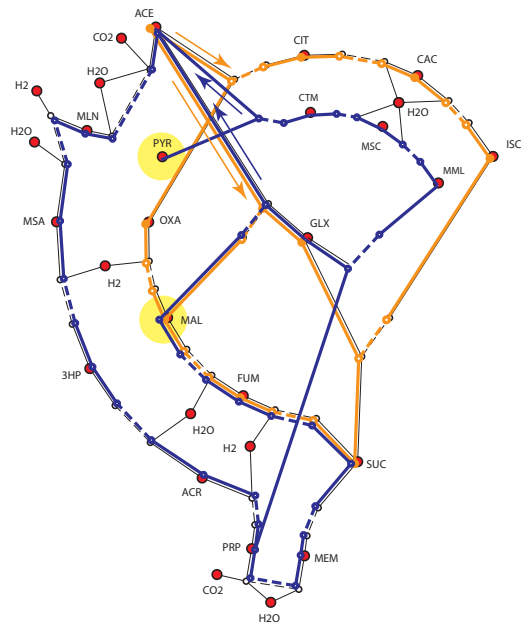


FIG. 9: The 3-hydroxypropionate bicycle (blue) and the glyoxylate shunt (orange) compared. Directions of flow are indicated by arrows on the links to acetate (ACE). The common core that enables flux recycling in both pathways is the aldol reaction between glyoxylate (GLX), acetate, and malate (MAL). The four other aldol reactions (labeled by their cleavage direction) are from isocitrate (ISC), methyl-malate (MML), citrate (CIT), and citramalate (CTM). Malate is a recycled network catalyst in both pathways. Carbon is fixed in the 3HP-bicycle as pyruvate (PYR), so the cycle only becomes autocatalytic if pyruvate can be converted to malate through anaplerotic (rTCA) reactions.

Fewer aldol reactions are possible among intermediates of the rTCA cycle and their homologues such as methyl-malate or citramalate in other carbon-fixation pathways, but all possibilities are indeed used either in intermediary metabolism or in carbon fixation. Fig. 9 shows the 3-hydroxypropionate bicycle and the closely-related glyoxylate shunt. In both pathways, the network topologies that regenerate all carbon flux or achieve autocatalysis are created by aldol reactions. The retention of carbon within the shunt appears to be a reason for its widespread distribution and frequent use [105, 135, 136], even when energetically more-efficient pathways such as the Krebs cycle exist as alternatives within organisms.

<sup>29</sup> The many parallel connections in networks such as the 3HP-bicycle or the pentose-phosphate network (see Fig. 5) make the problem of metabolite interconversion complex [61] in a different way than arises in the metabolic “bowtie”. Optimal conversion within the bowtie consists of finding common molecular “divisors” of input and output metabolites, and so can be seen even in the number theory of highly abstracted string chemistries [44]. The fact that short paths exist from most metabolites to a small set of building blocks is, in our interpretation, a reflection of the prior role of the core (where the building blocks are first created) in defining the possibilities for later anabolism and thus the metabolites reached by the bowtie.

### 5. Re-use of the direct C<sub>1</sub> reduction pathway and hybrid fixation strategies

A unique form of re-use is found for the sequence of reactions that directly reduce one-carbon (C<sub>1</sub>) groups on pterin cofactors. We have argued elsewhere [41] that even when a complete, autotrophic WL pathway is not present due to the loss of the oxygen-sensitive CODH/ACS enzyme, the direct C<sub>1</sub>-reduction sequence on pterins is often still present and being used as a partial fixation pathway. The reaction sequence supplies the diverse methyl-group chemistry mediated by S-adenosyl-methionine, and the direct synthesis of glycine and serine from methylene groups, reductant, and ammonia. These then serve as precursors to cysteine and tryptophan. The pathway may exist in either a complete (8-reaction) or a previously-unrecognized but potentially widespread (7-reaction) form that involves uptake on N<sup>5</sup> rather than N<sup>10</sup> of THF [41] (see Fig. 4.)

The widely distributed and diversified form of direct C<sub>1</sub> reduction functions much as auxiliary catabolic pathways function in mixotrophs [5], operating in parallel to an independent “primary” fixation pathway, with the primary and the direct-C<sub>1</sub> pathway supplying carbon to different subsets of core metabolites. In many cases where the CODH/ACS is lost, this loss disconnects the primary and direct-C<sub>1</sub> pathway segments, creating the novel feature of a *disjoint* carbon fixation pathway.<sup>30</sup> The existence of parallel fixation pathways in autotrophs had previously been recognized only in one (relatively late-branching)  $\gamma$ -proteobacterium, the uncultured endosymbiont of the deep-sea tube worm *Riftia pachyptila*, which was found to be able to use both the rTCA and CBB cycles [137]. In that case, however, the two pathways are not disjointed, but rather connected through intermediates in the glycolytic/gluconeogenic pathways. In addition, the capacity for using either cycle is thought to reflect an ability to adapt to variation in the availability of environmental energy sources, with an apparent up-regulation of the more efficient rTCA cycle under energy-poor conditions [137]. Our phylogenetic reconstruction [41], however, indicate that parallel disjoint pathways were the majority phenotype in the deep tree of life, in which a reductive C<sub>1</sub> sequence to glycine and serine is preserved in combination with with rTCA in Aquificales and Nitrospirae, with CBB in Cyanobacteria, with the 3HP bicycle in Chloroflexi (all bacteria), and with DC/4HB in Desulfurococcales and Acetolobales, and the 3HP/4HB cycle in Sulfolobales (all archaea). In contrast, the full WL pathway is found only in a subset of lin-

eages of bacteria (especially acetogenic Firmicutes) and archaea (methanogenic Euryarcheota).

Apparently as a result of the flexibility enabled by parallel carbon inputs to core metabolites, the direct C<sub>1</sub> reduction sequence is more universally distributed than any of the other loop-networks (whether paired with C<sub>1</sub> reduction or used as exclusive fixation pathways), or than the complete WL pathway. The status of the pterin-mediated sequence as a module appears more fundamental than its integration into the full WL pathway, and comparable to the arcs identified within rTCA, which may function as parts of fixation pathways or alternatively as anaplerotic extensions to other pathways. The two types of pathways also serve similar functional roles in our phylogenetic reconstruction of a root carbon-fixation phenotype, as the key components enabling and selecting the core anabolic precursors.

The reductive synthesis of glycine furnishes a potent reminder of the importance of taking evolutionary context into account when interpreting results from studies of metabolism. Historically much of our understanding of biochemistry comes from the study of human (or more generally mammalian) physiology, which can introduce biases. We noted above the example of the reductive citric acid cycle, which is sometimes called the “reverse” citric acid cycle even though it was ancestral to the oxidative form. Similarly, the “glycine cleavage system” (GCS) was originally studied in rat and chicken livers [138], before being recognized as phylogenetically widespread. The distribution of this system is now known to be nearly universal across the tree of life (with methanogens being the main systematic exception, for reasons explained elsewhere [41]), suggesting it was present already in the LUCA. The lipoyl-protein based system has long been known to be fully reversible [138–140], and has nearly neutral thermodynamics at physiological conditions [74]. Thus, the LUCA could have this system either to synthesize or to cleave glycine. From this perspective the former possibility (synthesis) seems a more likely interpretation, even without additional data. Absent the interpretation bias from mammalian physiology, the choice between these alternatives might have become clear much sooner.

### C. A coarse-graining of carbon-fixation pathways

We can combine all the previous observations on modularity in carbon-fixation – the sharing of arcs between loop pathways, the re-use of TCA and reductive C<sub>1</sub> sequence to complete the set of anabolic pillars – to perform a “coarse-graining” of carbon-fixation. Combining the decomposition of Fig. 7 with the gluconeogenic and WL pathways in Fig. 5, we may list the seven modules from which all known autotrophic carbon-fixation pathways are assembled: 1) direct one-carbon reduction on folates or related compounds, with or without the CODH/ACS terminal reaction of WL; 2) the short-molecule rTCA arc

<sup>30</sup> A secondary connection between the two components may exist in the form of oxidative conversion of 3-phosphoglycerate to serine. This connection may lead subsequently to the loss of direct-C<sub>1</sub> reduction as a fixation route, as in the proteobacteria, or it may release a constraint leading to change in pterin cofactor chemistry as in methanogens, discussed below.

from acetyl-CoA to succinyl-CoA; 3) the long-molecule rTCA arc from succinyl-CoA to citryl-CoA; 4) the gluconeogenic/reductive pentose-phosphate pathway, with or without the RubisCO reaction of CBB; 5) the 3HP arc from acetyl-CoA to succinyl-CoA; 6) the long-molecule 4HB pathway from succinyl-CoA to acetoacetyl-CoA; 7) the glyoxylate-shunt/mesaconate pathway to citramalate, which is the long-molecule loop in the 3HP bicycle. Fig. 10 shows the summary of these modules at the pathway level, as well as their different combinations to form complete autotrophic carbon-fixation pathways.

The importance of including glycine in the set of anabolic pillars immediately becomes clear in this coarse-grained view. The general similarity among different carbon-fixation pathways increases significantly, while finer distinction between forms becomes possible. In particular, both of the pathways that have been most commonly discussed in the context of ancestral carbon-fixation and the origin of life, WL and rTCA [7, 27, 73, 88], separate into deep- and late-branching forms. The increased similarity of the deep-branching forms of these pathways suggests an underlying template that combines both WL and rTCA in a fully connected network. WL and rTCA differ from this linked network by single reactions associated either with energy (ATP) economy or oxygen (or perhaps other oxidant) sensitivity. Combining information on the synthesis, structural variation, ecology and phylogenetics of the pterin molecules upon which direct  $C_1$  reduction is performed similarly suggests a distinction between the acetogenic (bacterial) and methanogenic (archaeal) forms of WL associated with energy economy [41]. A “proto-tree” of carbon-fixation emerges from the pooling of these different observations, which in turn makes it possible to reconstruct a complete phylometabolic tree of carbon-fixation, as discussed in detail in section IIID below.

### 1. How the inventory of elementary modules has constrained innovation and evolution

The essential invariance across the biosphere of the seven sub-networks listed above allows us to represent all carbon-fixation phenotypes in terms of the presence or absence, connectivity, and direction of these basic modules. In this representation, metabolic innovation at the modular level retains the character of individual discrete events, even if the pathway segments involved incorporate multiple genes. In cases where multiple genes must be acquired to constitute a module, as in the innovation of the 4HP pathway, this innovation may take place at higher levels of metabolism (e.g. fermentative secondary metabolism), after which their incorporation as fixation pathways appears as a single innovation.

Because the module boundaries are defined by particular (often universal) molecular species (e.g., acetyl-CoA, succinyl-CoA, and ribulose-1,5-bisphosphate) it often remains true that innovation can be traced to the

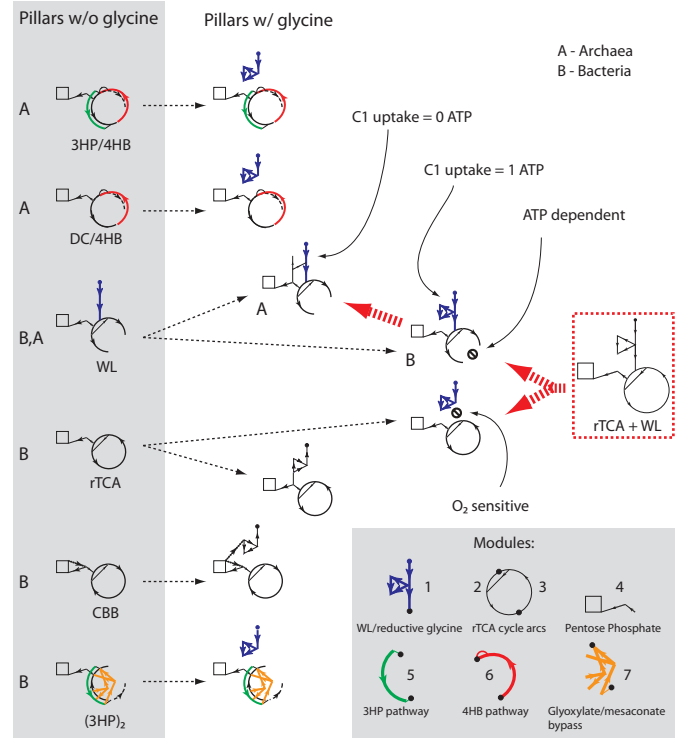


FIG. 10: Coarse-grained summary of carbon-fixation pathways. The left panel shows the six pathways as they are known from extensive laboratory characterization. Including glycine along with the anabolic pillars as the molecules that must be reached in carbon-fixation then adds resolution, allowing finer distinctions among forms and generally increasing their similarity. As a result, underlying evolutionary templates and patterns begin to emerge. The panel on the bottom right shows the modules from which all carbon-fixation pathways are constructed, as outlined in the main text.

change in single genes. This is true for the loss of the CODH/ACS from acetyl-CoA phenotypes, the innovation of RubisCO in CBB bacteria, or the loss of substrate-level phosphorylation to acetyl-CoA or succinyl-CoA in acetogens. A case with only slightly greater complexity is the apparently repeated, convergent evolution of an oxidative pathway to form serine from 3-phosphoglycerate (3PG), which involves three common and widely diversified reactions: a dehydrogenation, a reductive transamination, and a dephosphorylation.

At the module level, we may represent changes in carbon fixation pathways between closely-related phenotypes in terms of single connections, disconnections, or overall changes of direction within the subsets of the seven modules which are present. The change of direction within modules is usually complete, even if it is partial or intermediate at the level of whole pathways. An example is the switch from autotrophic rTCA to fermentative TCA using a reductive small-molecule arc and an oxidative large-molecule arc [91]. Such fermentative pathways

may alternate with fully oxidative TCA (Krebs) cycling, and they often occur in organisms that carry homologues to genes for both oxidative and reductive pathway directions [92–94].

An important exception to this pattern is the partial reversal of the formyl-to-methylene sequence on folates, between its carbon-fixation role and its role in the catabolic cleavage of glycine. We refer in Ref. [41] to the module formed by combining the GCS with the methylene-serine transferase as the *glycine cycle*. The combination of the complex free energy landscape provided by the folates [65] with the reversibility and nearly neutral thermodynamics of the glycine cycle [74, 138] permits a high degree of flexibility within this module. Carbon can enter either directly through CO<sub>2</sub>, through serine (from 3PG), or through glycine (from glyoxylate), and from any of these sources may be redirected to all of C<sub>1</sub> chemistry. The topology of the main reaction sequence is preserved in all these cases of reversal, though new enzymes or cofactors may be recruited to reverse some reactions.<sup>31</sup>

#### D. Reconstructed evolutionary history

##### 1. Phylogeny suggests little historical contingency of deep evolution within the modular constraints

The small number of modules that contribute to carbon fixation, and the even smaller number of “gateway” molecules that serve as interfaces between most of them, permit free recombination into many phenotypes satisfying the constraints of autotrophy. An important consequence of free recombination is that the external constraint (autotrophy) does not lock in dependencies within networks over separations larger than the modules themselves. Homology across intra-modular reaction sequences – especially if it is due to catalytic promiscuity – further weakens any lock-in effect created by selection for metabolic completeness. Through these mechanisms modularity promotes innovation-sharing [141] and rapid and reliable adaptation [18] to environmental conditions, but reduces standing variation among individuals sharing a common environment.

As we reviewed in Sec. III, distinct carbon-fixation pathway modules have very different couplings to the chemical environment. The genome distributions reported in Ref. [41] show that they also have very uneven

phylogenetic distribution. (For example, TCA arcs and intermediates, as well as direct C<sub>1</sub>-reduction, are nearly universally distributed, while the 3HP arcs are restricted to specific bacterial or archaeal clades living in alkaline environments.) Finally, we note that not all module combinations consistent with autotrophy have been observed in extant organisms.

By combining these observations it is possible to arrange autotrophic phenotypes on a graph according to their degree of similarity, and to assign environmental factors as correlates of phenotypic changes over most links. The graph projects onto a tree with very high parsimony and therefore almost no requirement to invoke either horizontal gene transfer or convergent evolution from distinct lineages. With a natural choice of root motivated by the overlap with bacterial and archaeal phylogeny, links become directed and environmental factors take on the interpretation of evolutionary causes. The lack of reticulation in a tree of innovations in autotrophy – at first surprising when compared to highly-reticulated gene phylogenies [142] covering the same period – becomes sensible as a record of invasion and adaptation to new chemical environments by organisms capable of maintaining little long-standing variation.

##### 2. A parsimony tree for autotrophic metabolism, and causation on links

The tree of autotrophic carbon-fixation phenotypes from Ref. [41] is shown in Fig. 11. All nodes in the tree satisfy the constraint that all five universal anabolic precursors plus glycine can be synthesized directly from CO<sub>2</sub>. We have defined parsimony by requiring single changes over links at the level of pathway modules, as explained above, rather than at the level of single genes, in cases where the two criteria differ. (This definition separates the evolution of genetic backgrounds, such as 4-hydroxybutyrate fermentation, from the events at which organisms came to rely on complete pathways for autotrophy.) A complete-parsimony tree for the known phenotypes is not possible, so we chose a tree in which the only violations are duplicate innovation of serine synthesis from 3-phosphoglycerate (common reactions and diversified enzyme families), and duplication or transfer of the short-molecule 3HP pathway (common environments).

The nodes in the tree of Fig. 11 are all phenotypes of extant organisms, with one important exception,<sup>32</sup>

<sup>31</sup> An example is the reversal of the complete rTCA cycle to the oxidative Krebs cycle. The electron donor in rTCA, reduced ferredoxin, is replaced by lipoic acid as an electron acceptor in the Krebs cycle, in the TPP-dependent oxidoreductase reaction. The enzymes catalyzing the retro-aldol cleavage of rTCA, which have undergone considerable re-arrangement even within the reductive world [89, 90], were further modified to the oxidative citryl-CoA synthetase.

<sup>32</sup> There is also one unimportant exception, which is the insertion of an acetogenic phenotype with a facultative oxidative pathway to serine at the root of the Euryarchaeota. Since methanogens use this pathway, and since an acetogenic pathway lacking oxidative serine synthesis is the most plausible ancestral form for all archaea as well as for Firmicutes within the bacteria, we infer that such an intermediate state did or does exist. This fixation pathway is consistent with forms observed in extant organisms,



which is the node between Aquificale branch and the Firmicute/Archaea branch. Aquificales and all phenotypes descending from them lack the CODH/ACS enzyme, while firmicutes and archaea lack one or more ATP-dependent acyl-CoA (citryl-CoA or succinyl-CoA) synthases. Therefore, if we seek a connected tree of life, two changes – the gain of one enzyme and loss of the other – are required to connect these branches. Since any organism lacking both enzymes could not fix carbon autotrophically, we have chosen the order of gain and loss so that the intermediate node has *both* the CODH/ACS and we expect that such a phenotype either will be discovered or will result from reclassification of genes in an existing organism.

and the acyl-CoA synthases. It therefore has both a complete WL pathway and an autocatalytic rTCA loop, connected through their shared intermediate acetyl-CoA. Losses (but not re-acquisitions) of either of these enzymes occur at multiple points on the tree, and both have likely explanations in either environmental chemistry or energetics. For this reason and several others given below, although a parsimony tree is (*a priori*) unrooted, we will regard the joint WL/rTCA phenotype as not only a bridging node but the root of the tree of autotrophs.

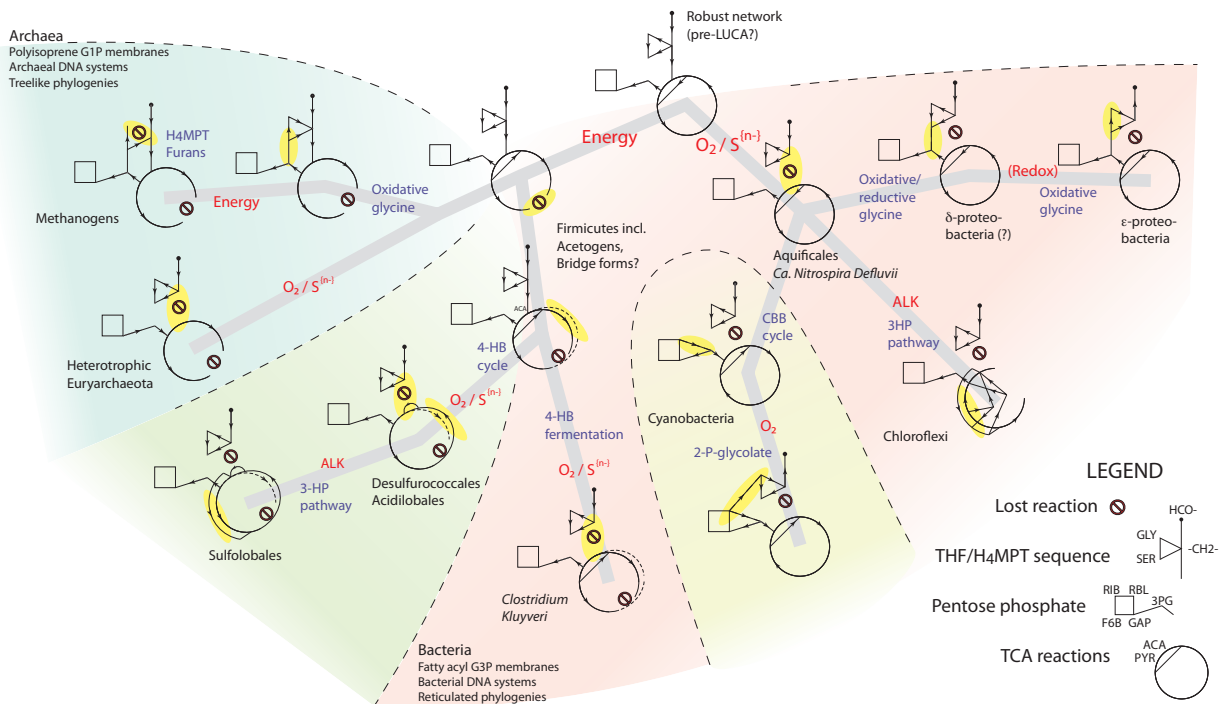


FIG. 11: A parsimony-based reconstruction of the innovations linking the major carbon-fixation phenotypes, from Ref. [41]. Nodes in the tree are autotrophic phenotypes, following the coarse-grained notation introduced in Fig. 10, and summarized in the legend. Grey links are transitions in the maximum-parsimony phylometabolic reconstruction, and yellow-highlighted regions in the diagrams are innovations following each link. Organism names or clades in which these phenotypes are found are given in black; fixation pathways innovated along each link are shown in blue, and imputed evolutionary causes are shown in red.  $S^{(n-)}$  refers to sulfides of different oxidation states. Dashed lines separate regions in which the clades by phylometabolic parsimony follow standard phylogenetic divisions. Abbreviations: formyl ( $\text{HCO}-$ ); methylene ( $-\text{CH}_2-$ ); acetyl-CoA (ACA); pyruvate (PYR); serine (SER); 3-phosphoglycerate (3PG); glyceraldehyde-3-phosphate (GAP); fructose-1,6-bisphosphate (F6B); ribose-phosphate (RIB); ribulose-phosphate (RBL); alkalinity (ALK). Arrows indicate reaction directions; dashed line connecting 3PG to SER indicate intermittent or bi-directional reactions.

In the evolution of carbon fixation from a joint WL/rTCA root, the primary division is between the loss of the CODH/ACS, resulting in rTCA loop-fixation phenotypes, and the loss of the acetyl-CoA or succinyl-CoA synthetases, resulting in acetogenic phenotypes.

Very low levels of oxygen permanently inactivate the CODH/ACS, so its loss is probable even under microaerobic conditions. Although the dominant mineral buffers for oxygen in the Archaeal remain a topic of significant uncertainty [143–146], it appears implausi-

ble that molecular oxygen was the toxin responsible for loss of the CODH/ACS much before the “Great Oxidation Event” (GOE).<sup>33</sup> Therefore the sensitivity of the CODH/ACS to sulfides or perhaps other oxidants (S. Ragsdale, pers. comm.) remains a possibly important factor in the early divergences of carbon fixation.

Alternatively, among strict WL-anaerobes, the loss of citryl-CoA or succinyl-CoA synthetase saves one ATP per carbon fixed, and all acetogenic phenotypes break rTCA cycling only through the loss of one or the other of these enzymes. We therefore interpret the loss of rTCA cycling as a result of selection for energy efficiency. The failure to regain either of these enzymes by acetogens which subsequently also lost the CODH/ACS is perhaps surprising given the inferred homology of the ancestral citryl-CoA and succinyl-CoA synthetases [89, 90], but explains the absence of rTCA cycling in either Firmicutes or any Archaea.

The remaining autotrophic phenotypes are derived from either rTCA cycling or acetogenesis in natural stages due to plausible environmental factors. Oxidative serine synthesis (from 3PG) is associated with the rise of the proteobacteria, whose differentiation in many features tracks the rise of oxygen and the transition to oxidizing rather than reducing environments. RubisCO and subsequently photorespiration arise within the cyanobacteria. The innovation of the 3HP bicycle from the malonate pathway arises within the Chloroflexi. In both Firmicutes (bacteria) and the crenarchaea, 4-hydroxybutyrate (or closely related 4-aminobutyrate) fermentation is more or less developed. Closure of the fermentative arcs to form a ring, again driven by elimination of the CODH/ACS [41] leads to the DC/4HB pathway in Crenarchaeota, which is then specialized in the Sulfolobales to the alkaline 3HP/4HB pathway. The Euryarchaeota are distinguished by the absence of an alternative loop-fixation pathway to rTCA, so that all members are either methanogens or heterotrophs.

Similarly, the innovation of the 3HP pathways, using biotin, emerges as a specialization to invade extreme but relatively rare environments. A particularly interesting case is the modification of folates in archaea, leading from THF in ancestral nodes to tetrahydromethanopterin in the methanogens, which enables initial fixation of formate (formed by hydrogenation of CO<sub>2</sub>) in an ATP-free system [41, 65]. The root position of rTCA explains the preservation of rTCA arcs both in reductive acetyl-CoA pathways, and in anaplerotic pathways for other fixation pathways, and the root position of direct C<sub>1</sub> reduction explains its near-universal distribution.

### 3. Parsimony violation and the role of ecological interactions

A tree is by construction a summary statistic for the relations among the phenotypes which are its leaves or internal nodes. It is not inherently a map of species descent, and takes on that interpretation only when common ancestry is shown to explain the conditional independence of branches given their (topological) parent nodes. This caution is especially important for the interpretation of Fig. 11, which shows high parsimony in the deepest branches where horizontal gene transfer is generally believed to have been most intense [51, 52]. We have argued that this behavior is consistent in a tree of successive optimal adaptations to varied environments, by organisms that could maintain little persistent variation. Violations of parsimony that are improbable by evolutionary convergence contain information about contact among historically separated lineages. Under this interpretation the separation is primarily environmental, with the subsequent contact identifying ecological co-habitation. The possible transfer of genes for the 3HP pathway is especially plausible, as the organisms involved may have shared the same extreme (alkaline) environments and been under common selection pressure, which when severe is known to accelerate rates of gene transfer [149, 150].

While our methods in Ref. [41] (flux-balance analysis of core networks) may be interpreted as producing either organism models or meta-metabolome models of consortia, the general agreement with robust phylogenetic signatures from many different genomic phylogenies [142, 151, 152] may still suggest a dominant role for vertical descent among autotrophic *organisms* (and not merely consortia) in the early evolution of carbon fixation.

### 4. A non-modern but plausible form of redundancy in the root node

The joint WL/rTCA network was introduced into Fig. 11 to produce a connected tree containing only autotrophic nodes. Our constraints in choosing it led to a kind of redundancy not found in extant fixation pathways. Either WL or rTCA alone is self-maintaining (in a modern organism) so a network that incorporates both is *redundantly* autocatalytic. While this is an important and speculative departure from all known phenotypes, it can be argued to reduce fragilities in both WL and rTCA under conditions of poor catalysis or unreliable regulation of anabolism. In that respect it is a more plausible phenotype for a universal ancestor than any modern network.

The enhanced robustness of the joint network follows from the interaction of short-loop and long-loop autocatalysis. The threshold for autocatalysis in the rTCA loop, fragile against parasitic side reactions or uncon-

<sup>33</sup> The GOE is usually dated at 2.5 GYA, though arguments exist for low levels of oxygen as much as 50-100 million years earlier [147, 148]. These may be relevant dates to compare to genetically estimated loss events in later branches of the Archaea or possibly in the Clostridia, but they are not plausible as dates for the first branching in the tree of Fig. 11.

strained anabolism, is supported and given a recovery mode when fed by an independent supply of acetyl-CoA from WL. In turn, the production of a sufficient concentration of folates to support direct  $C_1$  reduction, fragile if the long biosynthetic pathway is unreliable, is augmented by additional carbon fixed in rTCA. These arguments are topological, and do not make specific reference to whether the catalysts for the underlying reactions are enzymes. They may provide context for (perhaps multi-stage) models of transition from primordial mineral catalysis [68, 153] to the eventual support of carbon fixation by biomolecules.

Fig. 12 shows a numerical solution for the current flow through a minimal version of the joint WL/rTCA network, with lumped-parameter representations of parasitic side reactions and the net free energy of formation of acetate. (The exact rate equations used, and their interpretation, are provided in an App. A.) In the absence of a WL “feeder” pathway, rTCA has a sharp threshold for the maintenance of flux through the network as a function of the free energy of formation of its output acetate. The existence of such a sharp threshold depending on the rate of parasitism, below which the cycle supports *no* transport, has been one of the major sources of criticism of network-autocatalytic pathways as models for proto-metabolism [154]. When WL is added as a feeder, however, the threshold disappears, and some nonzero flux passes through the pathway at any positive free energy of formation of the outputs.

Chemical self-amplification, if it can be demonstrated experimentally, is the most plausible mechanism by which the biosphere can concentrate all energy flows and material cycles through a small, stable set of organic compounds. It supplies the molecules that are within the loop – and secondarily those that are made from loop intermediates – above the concentrations they would have in a Gibbs equilibrium distribution, as a result of flow through the network. The fact that self-amplification is permitted to act in the model of Fig. 12, even below the chemical-potential difference where the rTCA loop alone is self-sustaining, provides a mechanism by which the loop intermediates could have been provided in excess supply in the earliest stages of the emergence of metabolism. We return in Sec. VI to a related form of robustness and selection, which applies as anabolic pathways begin to form from loop intermediates.

### E. The rise of oxygen, and changes in the evolutionary dynamics of core metabolism

The limits of the phylometabolic tree we show in Fig. 11 fall on a horizon that coincides with the rise of oxygen. More precisely: we do not show branches that phylogenetically trace lineage divisions later than this horizon, because no known divisions in carbon fixation distinguish such later branches. Many of the late branches contain only heterotrophs, and to the extent

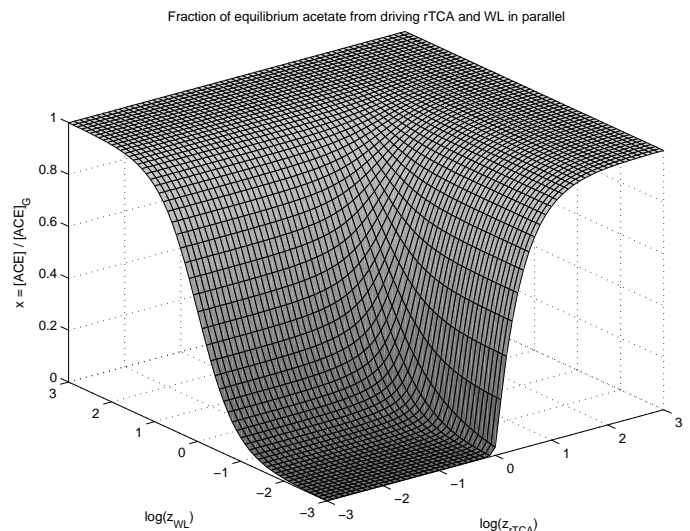


FIG. 12: Graph of solutions to Eq. (A44) from App. A is shown versus base-10 logarithms of  $z_{rTCA}$  and  $z_{WL}$ . The quantity  $x$  on the  $z$ -axis is the fraction of the acetate concentration  $[ACE]$  relative to the value it would take in an equilibrium ensemble with carbon dioxide, reductant, and water. The value  $x = 1$  corresponds to an asymptotically zero impedance of the chemical network, compared to the rate of environmental drain. The parameter  $z_{rTCA}$  is a monotone function of the non-equilibrium driving chemical potential to synthesize acetate, and  $z_{WL}$  measures the conductance of the “feeder” WL pathway. At  $z_{WL} \rightarrow 0$ , the WL pathway contributes nothing, and the rTCA network has a sharp catalytic threshold at  $z_{rTCA} = 1$ . For nonzero  $z_{WL}$ , the transition is smoothed, so some excess population of rTCA intermediates occurs at any driving chemical potential.

that post-oxygen lineage divisions follow divisions in metabolism, they are divisions in forms of heterotrophy. The rise of oxygen seems to have put an end to innovation in carbon fixation, and led to a florescence of innovation in carbon sharing.<sup>34</sup>

On the same horizon, the high parsimony of the tree we have shown ends, and it becomes necessary to explain complex metabolisms as a consequence of transfer of metabolic modules among clades in which they had evolved separately. We no longer expect that it would be possible to explain – and to some extent to predict – these innovations given only constraints of chemistry and

<sup>34</sup> By “sharing” we refer to general exchanges in which organic compounds are re-used without *de novo* synthesis; we do not intend only symbiotic associations. At the level of aggregate-ecosystem net primary production, the exchange of organics with incomplete catabolism may, however, reduce the free energy cost of the *de novo* synthesis of biomass that supports a given level of phenotypic diversity or specialization, allowing ecologies of complementary specialists to partially displace ecologies of generalist autotrophs.

invasion of new geochemical environments. Instead, they rely chemically on ecologically determined carbon flows, and genetically on opportunities for transfer of genes or pathway segments. Therefore any explanation will require some explicit model of ecological dynamics, and may require invoking some accidents of historical contingency. This contrast of phylometabolic reconstructions, between later and earlier periods, illustrates our association of parsimony violation with the role of ecosystems and explicit contributions of multilevel dynamics to evolution.

It is perhaps counterintuitive, but we believe consistent, that the phylometabolic tree is more tree-like in the earlier era of more extensive single-gene lateral transfers, and becomes *less* tree-like and more reticulated, in the era of complex ecosystems enabled by oxygenic metabolisms, which may have come as much as 1.5 billion years later. For reticulation to appear in a tree of reconstructed metabolisms, it is necessary that variants which evolved independently – as we have argued, under distinct selection pressures – be maintained in new environments where they can be brought into both con-

tact and interdependence. The maintenance of standing variation is facilitated both by the evolution of more advanced mechanisms to integrate genomes and limit horizontal transfer, and by the greater power density of oxygenic metabolisms.

The *serine cycle* used by some methylotrophic proteobacteria, shown in Fig. 13, provides an example of the structure and complex inheritance of a post-oxygen, heterotrophic pathway. Methylotrophs possess both an  $H_4MPT$  system transferred from methanogenic archaea [155, 156], and a conserved THF system ancestral to the proteobacteria (and we argue, to the universal common ancestor). In methylotrophs,  $H_4MPT$  is primarily used for the oxidation of formaldehyde to formate, while THF can be used in both the oxidative direction as part of the demethylation of various reduced one-carbon compounds, and in the reduction of formate.  $C_1$  compounds are then assimilated either as  $CO_2$  in the CBB cycle, as methylene-groups and  $CO_2$  in the serine cycle or as formaldehyde in the ribulose monophosphate (RuMP) cycle, in which formaldehyde is attached to ribulose-5-phosphate to produce fructose-6-phosphate [157, 158].

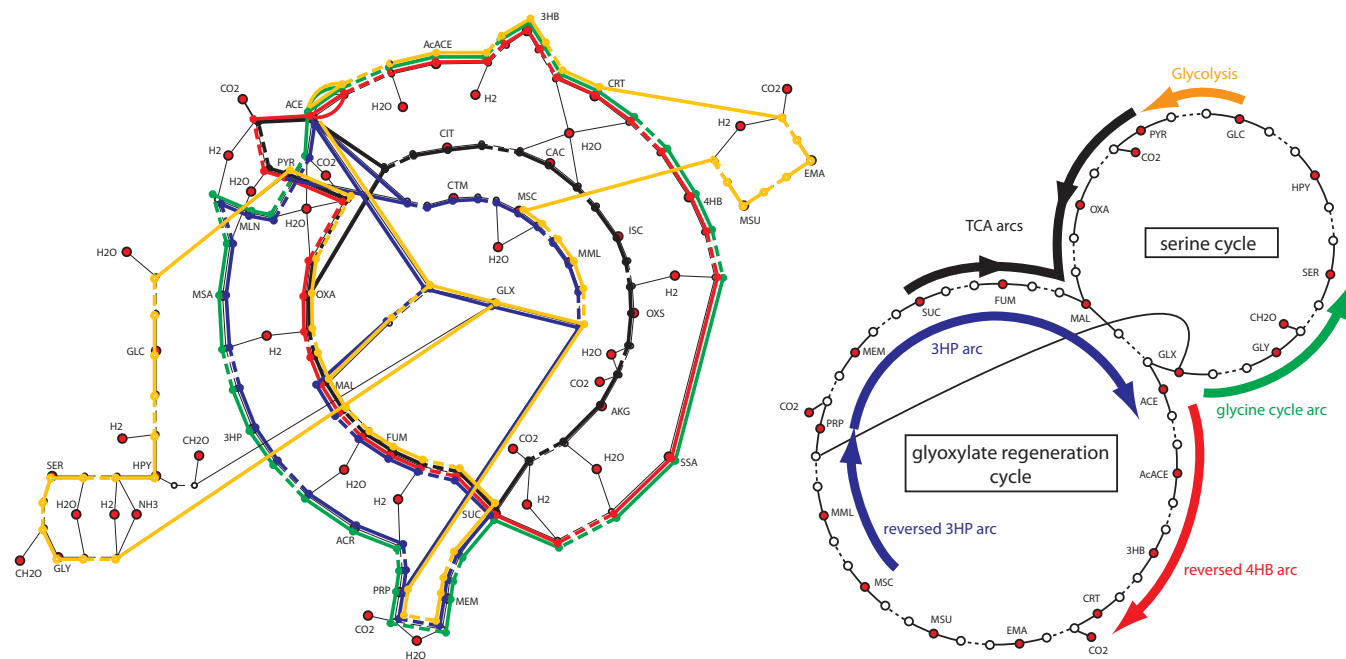


FIG. 13: The serine cycle/glyoxylate-regeneration cycle of methylotrophy. Left panel shows the stoichiometric pathway overlaid on the autotrophic loop pathways from Fig. 7. Right panel gives a projection of the serine cycle and glyoxylate regeneration cycle showing pathway directions; overlaps with the predecessor autotrophic pathways are labeled.

The full substrate network of the most complex assimilatory pathway of methylotrophy is a bicycle in which the serine cycle is coupled to the *glyoxylate regeneration cycle*. This full network employs segments of all four

loop-autotrophic pathways, as well as reactions in glycolysis, and part of the “glycine cycle”. Carbon enters the pathway at several points. Methylene groups enter through the glycine cycle, combining with glycine to form

serine. Serine is then deaminated and reduced to pyruvate, which is combined with a  $\text{CO}_2$  in a carboxylation to enter the core of TCA reactions. TCA arcs are performed reductively from pyruvate to malate, and oxidatively from succinate to malate, following the pattern of the 3HP pathway plus anaplerotic reactions from its output pyruvate. The short-molecule arc of 3HP is run as in the autotrophic carbon-fixation pathway starting from propionate, but part of the long-molecule arc of 3HP is reversed in the glyoxylate regeneration cycle. The 4HB pathway arc, transferred from archaea, is also reversed to feed this glyoxylate cycle, and is followed by a final additional carboxylation unique to this pathway [159, 160].

The serine/glyoxylate cycle of methylotrophy is a remarkable “Frankenstein’s monster” of metabolism, stitched together from parts of all pre-existing pathways, but requiring almost nothing new in its own local chemistry. Notably, the modules in this bacterial pathway which have been inherited from archaea are all reversed from the archaeal direction.

#### **F. Summary: Catalytic control as a central source of modularity in metabolism**

Focusing on the metabolic foundation of the biosphere – carbon-fixation and its interface with anabolism – we have seen many examples of how catalytic control is a central organizing principle in metabolism. The most complex and conserved reaction mechanisms in carbon-fixation often have unique (often very elaborate) metal centers and cofactors associated to them, reflecting the difficulty of the catalytic problem being solved. Not surprisingly, these reactions form the boundaries at which the various modules making up carbon-fixation are connected. As a result, these module boundaries form some of the strongest long-term constraints on evolution. They act as “turnstiles” along which the flow of carbon into the biosphere is redirected upon biogeochemical perturbations, resulting in the deepest structure in the tree of life.

The catalytic control of classes of organic reactions also leads to a secondary source of modularity, the locking in of various core pathways in the elaboration of downstream intermediary metabolism. The most striking example of this is that across the modern biosphere all anabolic pathways originate in only a very small number of molecules, mostly within the TCA cycle, even though a variety of different carbon-fixation strategies are used. The suggested interpretation is that much of intermediary metabolism had elaborated prior to the divergences in carbon-fixation. A related, but slightly different form of lock-in is found in the construction of methylotrophic pathways, which circumvents innovations in the catalytic control of difficult chemistry by re-using a wide range of parts from pre-existing carbon-fixation pathways.

### **IV. COFACTORS, AND THE EMERGENCE AND CENTRALIZATION OF METABOLIC CONTROL**

Cofactors form a unique and essential class of components within biochemistry, both as individual molecules and as a distinctive level in the control over metabolism. In synthesis and structure they tend to be among the most complex of the metabolites, and unlike amino acids, nucleotides, sugars and lipids, they are not primary structural elements of the macromolecular components of cells. Instead, cofactors provide a limited but essential inventory of functions, which are used widely and in a variety of macromolecular contexts. As a result they often have the highest connectivity (forming topological “hubs”) within metabolic networks, and are required in conjunction with key inputs or enzymes [161–163] to complete the most elaborate metabolisms.

In this section we will discuss how cofactors determine and regulate the scope of organic reactions in biochemistry, and how as focal points of selection they have been important in the large-scale structure of evolution. In understanding the role of cofactors in the emergence and evolution of metabolism, two consequences of their functional roles are essential to acknowledge. First, as we have discussed, cofactor functions are central in going from the short-loop network autocatalysis that would have been abiotically favored with proper mineral supports, to the long-loop network autocatalysis upon which all life today rests. As we will see, the most structurally complex cofactors are associated with the most catalytically complex functions within carbon-fixation, and thus form the most elaborate long-loop feedback closures at the substrate level. Second, because cofactor functions are associated with kinetic bottlenecks within metabolism, their inventory of functions form strong long-term constraints on the evolution of new pathways, so innovations in cofactor synthesis can have dramatic effects on the large-scale structure of evolution.

#### **A. Introduction to cofactors as a group, and why they define an essential layer in the control of metabolism**

##### *1. Cofactors as a class in extant biochemistry*

The biosynthesis of cofactors involves some of the most elaborate and least understood organic chemistry used by organisms. The pathways leading to several major cofactors have only very recently been elucidated or remain to be fully described, and their study continues to lead to the discovery of novel reaction mechanisms and enzymes that are unique to cofactor synthesis [164–166]. While cofactor biosynthetic pathways often branch from core metabolic pathways, their novel reactions may produce special bonds and molecular structures not found elsewhere in metabolism. These novel bonds and structures

are generally central in their catalytic functions.

Structurally, many cofactors form a class in transition between the core metabolites and the oligomers. They contain some of the largest directly-assembled organic monomers (pterins, flavins, thiamin, tetrapyrroles), but many also show the beginnings of polymerization of standard amino acids, lipids or ribonucleotides. These may be joined by the same phosphate ester bonds that link RNA oligomers or aminoacyl-tRNA, or they may use distinctive bonds (*e.g.* 5'-5' esters) found only in the cofactor class [167].

The polymerization exhibited within cofactors is distinguished from that of oligomers by its heterogeneity. Srinivasan and Morowitz [40] have termed them “chimeromers”, because they often include monomeric components from several molecule classes. Examples are coenzyme-A, which includes several peptide units and an ATP; folates, which join a pterin moiety to para-aminobenzoic acid (PABA); quinones, which join a PABA derivative to an isoprene lipid tail; and a variety of cofactors assembled on phosphoribosyl-pyrophosphate (PRPP) to which RNA “handles” are esterified.

We may understand the border between small and large molecules, where most cofactors are found, as more fundamentally a border between the use of heterogeneous organic chemistry to encode biological information in covalent structures, and the transition to homogeneous phosphate chemistry, with information carried in sequences or higher-order non-covalent structures. The chemistry of the metabolic substrate is mostly the chemistry of organic reactions. Phosphates and thioesters may appear in intermediates, but their role generally is to provide energy for leaving groups, enabling formation of the main structural bonds among C, N, O, and H. One of the striking characteristics scales in metabolism is that its organic reactions, the near-universal mode of construction for molecules of 20 to 30 carbons or less, cease to be used in the synthesis of larger molecules.<sup>35</sup> Large oligomeric macromolecules are almost entirely synthesized using the dehydration potential of phosphates [170] to link monomers drawn from the inventory [39] of small core metabolites. Many cofactors have structure of both kinds, and they are the smallest molecules that as a class commonly use phosphate esters as permanent elements of structure [171].

Finally, cofactors are distinguished by structure-function relations determined mostly at the single-molecule scale. The monomers that are incorporated into macromolecules are often distinguished by general properties, and only take on more specific functional roles that depend strongly on location and context [172, 173]. In contrast, the functions of cofactors are specific, of-

ten finely tuned by evolution [65], and deployable in a wide range of macromolecular contexts. Usually they are carriers or transfer agents of functional groups or reductants in intermediary metabolism [174]. Nearly half of enzymes require cofactors as coenzymes [171, 174]. If we extend this grouping to include chelated metals [175, 176] and clusters, ranging from common iron-sulfur centers to the elaborate metal centers of gas-handling enzymes [80, 120], more than half of enzymes require coenzymes or metals in the active site.

The universal reactions of intermediary metabolism depend on only about 30 cofactors [174] (though this number depends on the specific definition used). Major functional roles include 1) transition-metal-mediated redox reactions (heme, cobalamin, the Nickel tetrapyrrole F<sub>430</sub>, chlorophylls<sup>36</sup>), 2) transport of one-carbon groups that range in redox state from oxidized (biotin for carboxyl groups, methanofurans for formyl groups) to reduced (lipoic acid for methylene groups, S-adenosyl methionine, coenzyme-M and cobalamin for methyl groups), with some cofactors spanning this range and mediating interconversion of oxidation states (the folate family interconverting formyl to methyl groups), 3) transport of amino groups (pyridoxal phosphate, glutamate, glutamine), 4) reductants (nicotinamide cofactors, flavins, deazaflavins, lipoic acid, and coenzyme-B), 5) membrane electron transport and temporary storage (quinones), 6) transport of more complex units such as acyl and aminoacyl groups (panthetheine in CoA and in the acyl-carrier protein (ACP), lipoic acid, thiamine pyrophosphate), 7) transport of dehydration potential from phosphate esters (nucleoside di- and tri-phosphates), and 8) sources of thioester bonds for substrate-level phosphorylation and other reactions (panthetheine in CoA).

## 2. Roles as controllers, and consequences for the emergence and early evolution of life

Cofactors fill roles in network or molecular catalysis below the level of enzymes, but they share with all catalysts the property that they are not consumed by participating in reactions, and therefore are key loci of control over metabolism. Cofactors as transfer agents are essential to completing many network-catalytic loops. In association with enzymes, they can create channels<sup>37</sup> and ac-

<sup>35</sup> Even siderophores, among the most complex of widely-used organic compounds, are often elaborations of functional centers that are small core metabolites, such as citrate [168, 169].

<sup>36</sup> It is natural in many respects to include Ferredoxins (and related flavodoxins) in this list. Although not cofactors by the criteria of size and biosynthetic complexity, these small, widely-diversified, ancient, and general-purpose Fe<sub>2</sub>S<sub>2</sub>, Fe<sub>3</sub>S<sub>4</sub>, and Fe<sub>4</sub>S<sub>4</sub>-binding polypeptides are unique low-potential (high-energy) electron donors. Reduced Ferredoxins are often generated in reactions involving radical intermediates in iron-sulfur enzymes, described below in connection with electron bifurcation.

<sup>37</sup> An example is the role of cobalamin as a C<sub>1</sub> transfer agent to the Nickel reaction center in the acetyl-CoA synthase from a corrinoid iron-sulfur protein [177–179].



tive sites<sup>38</sup>, and thus they facilitate molecular catalysis. Through the limits in their own functions or in the functional groups they transport through networks, they may impose constraints on chemical diversity or create bottlenecks to evolutionary innovation. The previous sections have shown that many module boundaries in carbon fixation and core metabolism are defined by idiosyncratic reactions, and we have noted that many of these idiosyncrasies are associated with specific cofactor functions.

Cofactors, as topological hubs, and participants in reactions at high-flux boundaries in core and intermediary metabolism, are focal points of natural selection. The adaptations available to key atoms and bonds include altering charge or pKa, changing energy level spacing through non-local electron transport, or altering orbital geometry through ring strains. Divergences in low-level cofactor chemistry may alter the distribution of functional groups and thereby change the global topology of metabolic networks,<sup>39</sup> and some of these changes map onto deep lineage divergences in the tree of life.

Most research on the origin of life has focused either on the metabolic substrate [6, 180] or catalysis by RNA [181], but we believe the priority of cofactors deserves (and is beginning to receive) greater consideration [182, 183]. In the expansion of metabolic substrates from inorganic inputs, the pathways to produce even such complex cofactors as folates *et alia* are comparable in position and complexity to those for purine RNA, while some for functional groups such as nicotinamide [182] or chorismate are considerably simpler. Therefore, even though it is not known what catalytic support or memory mechanisms enabled the initial elaboration of metabolism, any solutions to this problem should also support the early emergence of at least the major redox and C- and N-transfer cofactors. Con-

<sup>38</sup> An example is the role of TPP as the reaction center in the pyruvate-ferredoxin oxidoreductase (PFOR), which lies at the end of a long electron-transport channel formed by Fe-S clusters [84].

<sup>39</sup> A well-understood example is the repartitioning of C<sub>1</sub> flux from methanopterins versus folates [41, 65]. The same adaptation

versely, the pervasive dependence of biosynthetic reactions on cofactor intermediates makes the expansion of protometabolic networks most plausible if it was supported by contemporaneous emergence and elaboration of cofactor groups. In this interpretation cofactors occupy an intermediate position in chemistry and complexity, between the small-metabolite and oligomer levels [182]. They were the transitional phase when the reaction mechanisms of core metabolism came under selection and control of organic as opposed to mineral-based chemistry, and they provided the structured foundation from which the oligomer world grew.

We argue next that a few properties of the elements have governed both functional diversification and evolutionary optimization of many cofactors, especially those associated with core carbon-fixation. We focus in particular on heterocycles with conjugated double bonds incorporating nitrogen, and on the groups of functions that exploit special properties of bonds to sulfur atoms.

## B. The cofactors derived from purine RNA

Most of the cofactors that use heterocycles for their primary functions have biosynthetic reactions closely related to those for purine RNA. These reactions are performed by a diverse class of cyclohydrolase enzymes, which are responsible for the key ring-formation and ring-rearrangement steps. The cyclohydrolases can split and reform the ribosyl ring in PRPP, jointly with the 5- and 6-membered rings of guanine and adenine. Five biosynthetically related cofactor groups are formed in this way. Four of these – the folates, flavins, deazaflavins and thiamin – are formed from GTP, as shown in Fig. 14.

that enables formylation of methanopterins within an exclusively thioester system, where the homologous folate reaction requires ATP, reduces the potential for methylene-group transfer, and necessitates the oxidative formation of serine from 3PG in methanogens, which is not required of acetogens.

**Folates:** The folates are structurally most similar to GTP, but have undergone the widest range of secondary specializations, particularly in the Archaea. They are primarily responsible for binding C<sub>1</sub> groups during reduction from formyl to methylene or methyl oxidation states, and their secondary diversifications are apparently results of selection to tune the free-energy landscape of these oxidation states.

**Flavins and deazaflavins:** The flavins are tricyclic compounds formed by condensation of two pterin groups, while deazaflavins are synthesized through a modified version of this pathway, in which one pterin group is replaced by a benzene ring derived from choris-

mate. Flavins are general-purpose reductants, while deazaflavins are specifically associated with methanogenesis.

**Thiamin:** Thiamine combines a C-N heterocycle common to the GTP-derived cofactors with a thiazole group (so incorporating sulfur), and shares functions with both the purine cofactor group and the alkyl-thiol group reviewed in the next subsection.

**Histidine:** The last “cofactor” in this group is the amino acid histidine, synthesized from ATP rather than GTP but using similar reactions. Histidine is a general acid-base catalyst with unique pKa, which in many ways functions as a “cofactor in amino acid form” [40].

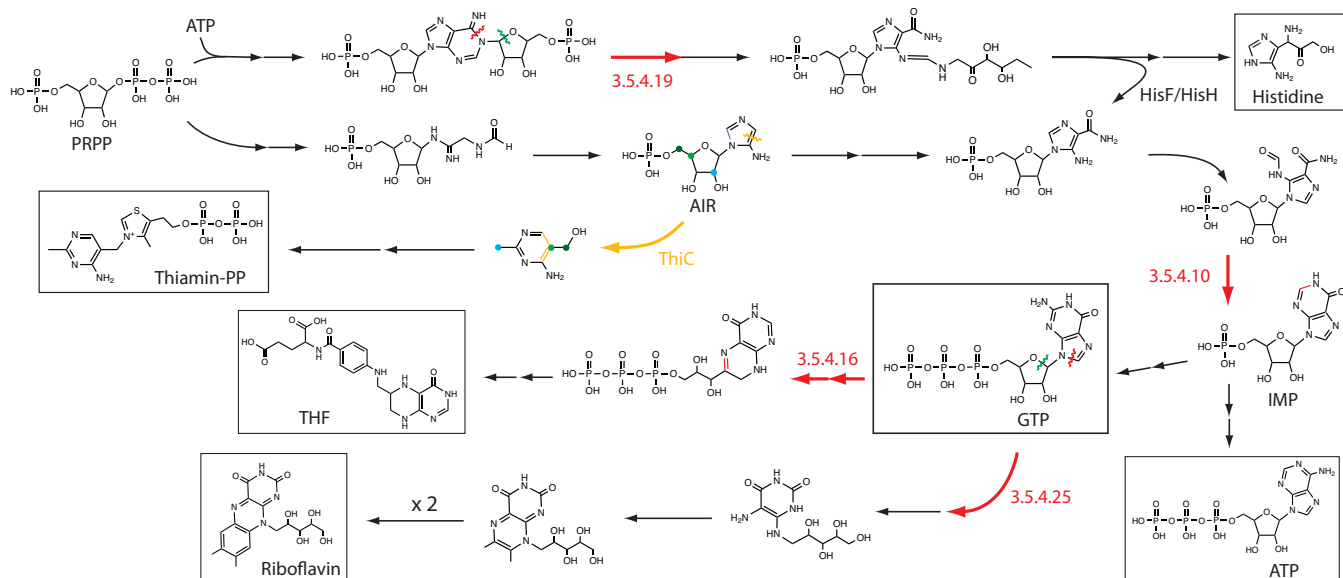


FIG. 14: Key molecular re-arrangements in the network leading from AIR to purines and the purine-derived cofactors. The 3.5.4 class of cyclohydrolases (red) convert FAICAR to IMP (precursor to purines), and subsequently convert GTP to folates and flavins by opening the imidazole ring. Acting on the 6-member ring of ATP and on a second attached PRPP, the enzyme 3.5.4.19 initiates the pathway to histidinol. The thiamine pathway, which uses the unclassified enzyme ThiC to hydrolize imidazole and ribosyl moieties, is the most complex, involving multiple group rearrangements (indicated by colored atoms). This complexity, together with the subsequent attachment of a thiazole group, lead us to place thiamine latest in evolutionary origin among these cofactors.

We will first describe in detail the remarkable role of the folate group in the evolutionary diversification of the Wood-Ljungdahl pathway, and then return to general patterns found among the purine-derived cofactors, and their placement within the elaboration of metabolism and RNA chemistry.

### 1. Folates and the central superhighway of $C_1$ metabolism

Members of the folate family carry  $C_1$  groups bound to either the  $N^5$  nitrogen of a heterocycle derived from GTP, an exocyclic  $N^{10}$  nitrogen derived from a para-aminobenzoic acid (PABA), or both. The two most common folates are tetrahydrofolate (THF), ubiquitous in bacteria and common in many archaeal groups, and tetrahydromethanopterin ( $H_4MPT$ ), essential for methanogens and found in a small number of late-branching bacterial clades. Other members of this family are exclusive to the archaeal domain and are structural intermediates between THF and  $H_4MPT$ . Two kinds of structural variation are found among folates, as shown in Fig. 15. First, only THF retains the carbonyl group of PABA, which shifts electron density away from  $N^{10}$  via the benzene ring, and lowers its pKa relative to  $N^5$  of the heterocycle. All other members of the family lack this carbonyl. Second, all folates besides THF incorporate one or two methyl groups that impede rotation between the pteridine and aryl-amine planes, changing the relative entropies of formation among different binding

states for the attached  $C_1$  [41, 65, 184].

Folates mediate a diverse array of  $C_1$  chemistry, various parts of which are essential in the biosynthesis of all organisms [65]. The collection of reactions, summarized in Fig. 4, has been termed the “central superhighway” of one-carbon metabolism. Functional groups supplied by folate chemistry, connected by interconversion of  $C_1$ -oxidation states along the superhighway, include 1) formyl groups for synthesis of purines, formyl-tRNA, and formylation of methionine (fMet) during translation, 2) methylene groups to form thymidilate, which are also used in many deep-branching organisms to synthesize glycine and serine, forming the ancestral pathway to these amino acids [41], and 3) methyl groups which may be transferred to S-adenosyl-methionine (SAM) as a general methyl donor in anabolism, to the acetyl-CoA synthase to form acetyl-CoA in the Wood-Ljungdahl pathway, or to coenzyme-M where the conversion to methane is the last step in the energy system of methanogenesis.

The variations among folates, shown in Fig. 15, leave the charge, pKa and resulting C-N bond energy at  $N^5$  roughly unaffected, while the  $N^{10}$  charge, pKa, and C-N bond energy change significantly across the family. This charge effect, together with entropic effects due to steric hindrance from methyl groups, can sharply vary the functional roles that different folates play in anabolism.

The biggest difference lies between THF and  $H_4MPT$ . In THF, the  $N^{10}$  pKa is as much as 6.0 natural-log units lower than that of  $N^5$  [185]. The resulting higher-energy

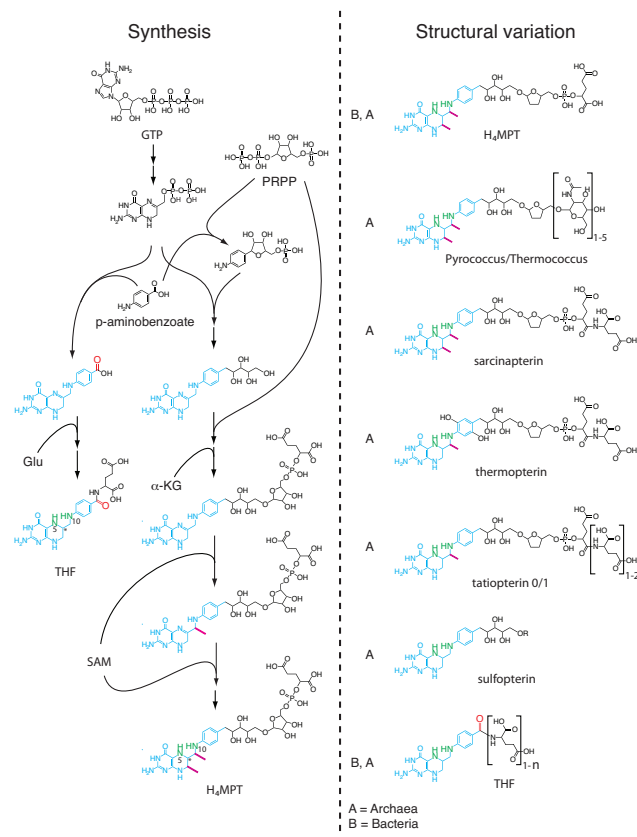


FIG. 15: Structural variants among cofactors in the folate family, shown with the biosynthetic pathways that produce these variations. Pteridine and benzene groups shown in blue, and methyl groups that regulate steric hindrance shown in red.

C-N bond cannot be formed without hydrolysis of one ATP, either to bind formate to N<sup>10</sup> of THF, or to cyclize N<sup>5</sup>-formyl-THF to form N<sup>5</sup>,N<sup>10</sup>-methenyl-THF (see Fig. 4).<sup>40</sup> After further reduction, the resulting methylene is readily transferred to lipoic acid to form glycine and serine, in what we have termed the “glycine cycle” [41] (the lipoyl-protein based cycle on the right in Fig. 4).

In contrast, in H<sub>4</sub>MPT the difference in pK<sub>a</sub> between N<sup>10</sup> and N<sup>5</sup> is only 2.4 natural-log units. The lower C-N<sup>10</sup> bond energy permits spontaneous cyclization of N<sup>5</sup>-formyl-H<sub>4</sub>MPT, following (also ATP-independent) transfer of formate from a formyl-methanofuran cofactor. Through this sequence, methanogens fix formate in an ATP-independent system using only redox chemistry.

<sup>40</sup> This reaction is the mirror image of the cyclization of N<sup>10</sup>-formyl-THF. This latter reaction is spontaneous. We will argue below that the alternative cyclization from N<sup>5</sup>-formyl-THF, previously only recognized as a salvage pathway [186], may reflect an unrecognized function of the cycloligase as an enzyme for ATP-dependent formate incorporation.

The initial free energy to attach formate to methanofuran is provided by the terminal methane released in methanogenesis (the Co-M/Co-B cycle in Fig. 4). The resulting downstream methylene group, however, has too little energy as a leaving group to transfer to an alkyl-thiol cofactor, so methanogens sacrifice the ability to form glycine and serine by direct reduction of formate.

The reconstructed ancestral use of the 7-9 reactions in Fig. 4 is to reduce formate to acetyl-CoA or methane. However, the reversibility of many reactions in the sequence, possibly requiring substitution of reductant/oxidant cofactors, allows folates to accept and donate C<sub>1</sub> groups in a variety of oxidation states, from and into many pathways including salvage pathways. Methylophilic proteobacteria which have obtained H<sub>4</sub>MPT through horizontal gene transfer [156, 157] may run the full reaction sequence in reverse. They may use either H<sub>4</sub>MPT to oxidize formaldehyde or THF to oxidize various methylated C<sub>1</sub> compounds, in both cases leading to formate, or other intermediary oxidation states (from THF) as inputs to anabolic pathways. In many late-branching bacteria, some archaea, and eukaryotes, the THF based pathway may run in part oxidatively and in part reductively, through connections to either gluconeogenesis/glycolysis or glyoxylate metabolism. In these organisms serine (derived through oxidation, amination and dephosphorylation from 3-phosphoglycerate) or glycine (derived through amination of glyoxylate) become the sources of transferable methyl groups in anabolism. This versatility has preserved the folate pathway as an essential module of biosynthesis in all domains of life, and at the same time has made it a pivot of evolutionary variation.

## 2. Refinement of folate-C<sub>1</sub> chemistry maps onto lineage divergence of methanogens

The structural and functional variation within the folate family illustrates the way that selection, acting on cofactors, can create large-scale re-arrangements in metabolism, enabling adaptations that are reflected in lineage divergences. The free-energy cascade described in the last section, linking ATP hydrolysis, the charge and pK<sub>a</sub> of the N<sup>10</sup> nitrogen, and the leaving-group activity of the resulting bound carbon for transfer to alkyl-thiol cofactors or other anabolic pathways, is a fundamental long-range constraint of folate-C<sub>1</sub> chemistry. A comparative analysis of gene profiles in pathways for glycine and serine synthesis, explained in Ref. [41], shows that while the constraint cannot be overcome, its impact on the form of metabolism can vary widely depending on the structure of the mediating folate cofactor.

The annotated role for ATP hydrolysis in WL autotrophs is to attach formate to N<sup>10</sup> of THF, initiating the reduction sequence. However, many deep-branching bacteria and archaea show no gene for this reaction, while multiple lines of evidence indicate that THF nonetheless

functions as a carbon-fixation cofactor in these organisms [41]. In almost all cases where an ATP-dependent  $N^{10}$ -formyl-THF synthase is absent, an ATP-dependent  $N^5$ -formyl-THF cycloligase [186, 187] is found. This is another case where a broad evolutionary context allows an alternate interpretation.  $N^5$ -formyl-THF cycloligase was originally discovered in mammalian systems, where its function has been highly uncertain and hypothesized to be the salvage mechanism as part of a futile cycle [186, 187], before being found to be widespread across the tree of life [41]. If we deduce by reconstruction, however, that ancestral folate chemistry operated in the fully reductive direction, and that in  $H_4MPT$  systems formate is attached at the  $N^5$  position, while in THF systems formate is attached at the  $N^{10}$  position, the widespread distribution of the cycloligase takes on a different meaning. It is plausible that the  $N^5$ -formyl-THF cycloligase allows a formate incorporation pathway that is an evolutionary intermediate between the commonly recognized pathway using THF and its evolutionary derivative using  $H_4MPT$  (see Fig. 4). The ATP-dependent cycloligase produces  $N^5, N^{10}$ -methenyl-THF from  $N^5$ -formyl-THF, which may potentially form spontaneously due to the higher  $N^5$ -pKa [187]. ATP hydrolysis is thus specifically linked to the  $N^{10}$ -carbon bond which is the primary donor for carbon groups from folates. Methanogens, in contrast, escape the dependence on ATP hydrolysis by decarboxylating PABA before it is linked to pteridine to form methanopterin (see Fig. 15), but they sacrifice methyl-group donation from  $H_4MPT$  to most anabolic pathways, making methanogenesis viable only in clades that evolved the oxidative pathway to serine from 3-phosphoglycerate.

We noted in Sec. IIID that the elimination of one ATP-dependent acyl-CoA synthase in acetogens reduces the free energy cost of carbon fixation relative to rTCA cycling. The decoupling of the formate-fixation step on methanopterins from ATP hydrolysis is a further significant innovation, lowering the ATP cost for uptake of  $CO_2$ . This divergence of  $H_4MPT$  from THF, and a related divergence of deazaflavins from flavins (see Fig. 16), follow phylogenetically (and we believe, were responsible for) the divergence of the methanogens from other euryarcheota [41].

We regard this example as representative of the way that innovations in cofactor chemistry more generally mediated large-scale rearrangements in metabolism, and corresponding evolutionary (and ecological) divergences of clades. Another similar example comes from the quinones, a diverse family of cofactors mediating membrane electron transport [188]. Ref. [189] found that the synthetic divergence of mena- and ubiquinone follows the pattern of phylogenetic diversification within proteobacteria.  $\delta$ - and  $\epsilon$ -proteobacteria use menaquinone,  $\gamma$ -proteobacteria use both mena- and ubiquinone, and  $\alpha$ - and  $\beta$ -proteobacteria use only ubiquinone. Because mena- and ubiquinone have different midpoint potentials, it was suggested that their distribution reflects changes in environmental redox state as the proteobacteria diver-

sified during the rise of oxygen [189, 190]. Such phylogenetic divergences may alternatively be thought of as divergences driven by the closure of more advantageous long-loop feedback cycles.

### 3. Relation of the organic superhighway to minerals

A very wide range of circumstantial arguments has been made for the emergence of biochemistry from the reduced-mineral/seawater chemistry of hydrothermal vents. These include: detailed accounts of the capacity of a range of geochemical energy systems to support extant life [25, 28],<sup>41</sup> detailed similarities between transition-metal/sulfide mineral unit cells and metallo-enzyme active sites [68, 119, 191], the widespread use of radical mechanisms in assembly of metal-center enzymes [120], and the more general presence of chelated metals in ubiquitous and conserved cofactors and enzymes (particularly tetrapyrroles and ferredoxins), the richness of vent environments in geometry, surface catalysis [88, 192, 193], thermal and pH gradients, and the greater similarity of the aqueous redox environment of hydrothermal fluids to biochemistry, than of atmospheric free-radical chemistry or the quenched ion chemistry in the interstellar medium [27, 66, 194, 195]. While these arguments still leave too many circumstantial steps to have created consensus that metabolism emerged through self-organization from geochemistry [154], among the many speculations about what was necessary for the first metabolism, the geochemical hypothesis is grounded in the widest array of relevant empirical evidence. The geochemical hypothesis has also been circumstantially supported by experimental evidence that minerals can catalyze reactions in the citric-acid cycle [71], and an extensive range of reductions [196, 197], including synthesis of acetyl-thioesters [56].

The distinctive features of biochemical  $C_1$  reduction are the attachment of formate to tuned heterocyclic or aryl-amine nitrogen atoms for reduction, and the transfer of reduced  $C_1$  groups to sulfhydryl groups (of SAM, lipoic acid, or CoM). In the mineral-origin hypothesis for direct reduction, the  $C_1$  were adsorbed at metals and either reduced through crystal oxidation [193] or by reductant in solution. The transfer of reduced  $C_1$  groups to alkyl-thiol cofactors may show continuity with reduction on metal-sulfide minerals. However, the mediation of reduction by

<sup>41</sup> A subset of the entries in Table 1 of Ref. [25], involving  $Fe^{2+}$  reduction or autotrophic methanogenesis, can be applied directly to early-earth environments. Many entries in their table of environments involve sulfates, nitrates, ferric iron, or small amount of molecular oxygen (the Knallgas reaction) as terminal electron acceptors. The organic conversions detailed in the paper remain a basis for habitability analysis, but plausible pathways in the Hadean will be limited by the alternative terminal electron acceptors.

nitrogens appears a distinctively biochemical innovation.

#### 4. Cyclohydrolases as the central enzymes in the family, and the resulting structural homologies among cofactors

The common reaction mechanism unifying the purine-derived cofactors is an initial hydrolysis of both purine and ribose rings performed by cyclohydrolases assigned EC numbers 3.5.4 (see Fig. 14). These enzymes are responsible for the synthesis of inosine-monophosphate (IMP, precursor to AMP and GMP) from 5-formamidoimidazole-4-carboxamide ribonucleotide (FAICAR), for the first committed steps in the syntheses of both folates and flavins from GTP, and for the initial ring-opening step in the synthesis of Histidine from ATP and PRPP. Fig. 14 shows the key steps in the network synthesizing both purines and the pterins, folates, flavins, thiamine, and histidine.

The common function of the 3.5.4 cyclohydrolases is hydrolysis of rings on adjacent nucleobase and ribose groups, or the formation of cycles by ligation of ring fragments. In all cases, the ribosyl moieties come from phosphoribosyl-pyrophosphate (PRPP). In the synthesis of pterins from GTP and of histidinol from ATP, both a nucleobase cycle and a ribose are cleaved. In pterin synthesis, the imidazole of guanine and the purine ribose are cleaved. In histidine synthesis, the six-membered ring of adenine is cleaved (at a different bond than the one synthesized from FAICAR), and the ribose comes from a secondary PRPP.

By far the most complex synthesis in this family is that of thiamin from aminoimidazole ribonucleotide (AIR). This sequence begins with an elaborate molecular rearrangement, performed in a single step by the enzyme ThiC [166]<sup>42</sup>. While this enzyme is unclassified, and its reaction mechanism incompletely understood, it shares apparent characteristics with members of the 3.5.4 cyclohydrolases. As in the first committed steps in the synthesis of folates and flavins from GTP, both a ribose ring and a 5-member heterocycle are cleaved and subsequently (as in folate synthesis) recombined into a 6-member heterocycle. The complexity of this enzymatic mechanism makes a pre-enzymatic homologue to ThiC difficult to imagine, and suggests that thiamin is both of later origin, and more highly derived, than other cofactors in this family. This derived status is supported by the fact that the resulting functional role of thiamin is not performed on the pyrimidine ring itself, but rather on the thiazole ring to which it is attached, and which is likewise created in an elaborate synthetic sequence [166]. The reactions involving TPP do not directly create bonds to the

sulfur atom, but instead use the carbon between it and the positively charged nitrogen. It seems likely, however, that the sulfur indirectly contributes to the properties of that carbon, through some combination of electrostatic, resonance, or possibly ring-straining interactions.

Fig. 16 shows the detailed substrate re-arrangement in the sub-network leading from GTP to methanopterins, folates, riboflavin, and the archaeal deazaflavin F<sub>420</sub>. In the pterin branch, both rings of neopterin are synthesized directly from GTP, and an aryl-amine originating in PABA provides the second essential nitrogen atom. PABA is either used directly (in folates) or decarboxylated with attachment of a PRPP (in methanopterins) to vary the pKa of the amine. In contrast, the flavin branch is characterized by the integration of either ribulose (in riboflavin) or chorismate (in F<sub>420</sub>) to form the internal rings. Two 6,7-Dimethyl-8-(D-ribityl)lumazine are condensed to form riboflavin, whereas a single GTP with chorismate forms F<sub>420</sub>.

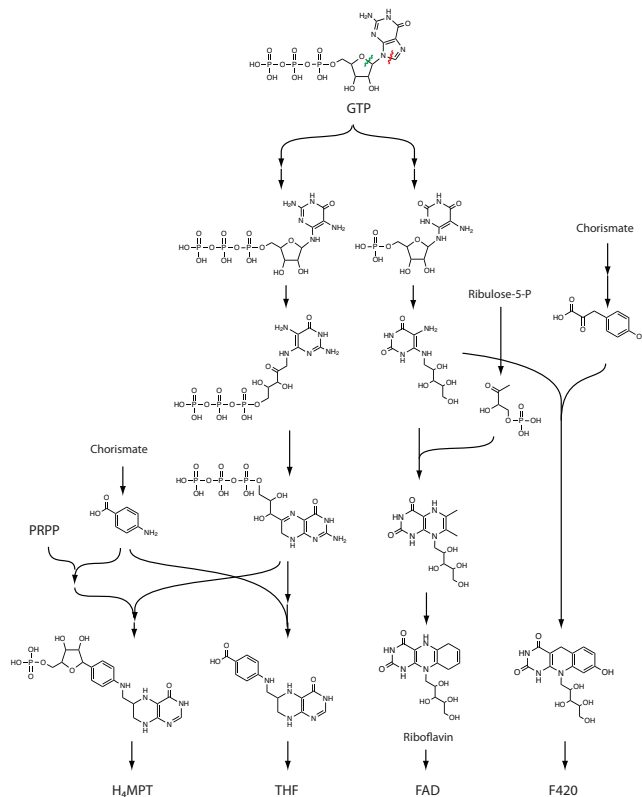


FIG. 16: The substrate modifications leading from GTP to the four major cofactors H<sub>4</sub>MPT, THF, riboflavin (in FAD) and the archaeal homologue deazaflavin F<sub>420</sub>. The branches indicating substrate diversification may also reflect an evolutionary lineage.

The cyclohydrolase reactions are the innovation enabling the biosynthesis of this whole family of cofactors, and importantly, of purine RNA itself. Except for TPP, the distinctions among purine-derived cofactors are minor secondary modifications on a background structured

<sup>42</sup> Eukaryotes use an entirely different pathway, in which the pyrimidine is synthesized from histidine and pyridoxal-5-phosphate [198]

by PRPP and C-N heterocycles. Chorismate, precursor to PABA and the unique source of single benzene rings in biochemistry, is the only other developed sub-network within metabolism, besides purine synthesis, on which this family draws. Flexibility in the ways that chorismate is modified to control electron density, and the way the benzene ring is combined with other heterocycles, contributes to the combinatorial elaboration within the family.

#### 5. *Placing the members of the class within the network expansion of metabolism*

The following observations suggest to us that most of the purine-derived cofactors (possibly excepting thiamin) were available contemporaneously with monomer purine RNA.

The current understanding of protein cyclohydrolases does not suggest other, simpler mechanisms by which similar reactions might first have been catalyzed.<sup>43</sup> However, at whatever stage catalysts capable of interconverting AIR, AICAR, FAICAR, and IMP first became available, there is no compelling reason to believe that pteridines were not formed contemporaneously. If the chorismate pathway (which begins in the sugar-phosphate network) had also arisen by that time, there is no compelling reason to believe that folates and flavins were not likewise available. Particularly if the early catalysts were primitive, opening reaction mechanisms at the level of the first three EC numbers but not restricting molecular substrates, it would be difficult to argue that molecules generally resembling this cofactor class could have been reliably *excluded* from a monomer-purine RNA world.<sup>44</sup>

Conversely, the patterns that characterize current metabolism as a recursive network expansion [161, 162] about inorganic inputs are most easily understood as a reflection of the organic-chemical possibilities opened by cofactors. Pterins, as donors of activated formyl groups, support (among other reactions) the synthesis of purines, forming a short autocatalytic loop. Similarly, flavins would have augmented redox reactions. Finally, it has long been recognized that acid/base catalysis is uniquely served by histidine, which has a  $pK_a \approx 6.5$  on the  $\epsilon$ -nitrogen, a property not found among any biological ribonucleotides (though possible for some substituted adenine derivatives) [202].

Within the class of GTP-derived cofactors, a substructure may perhaps be suggested: the dimer condensa-

tion that forms riboflavin is a hierarchical use of building blocks formed from GTP. Although simple and consisting of a single key reaction, this could reflect a later stage of refinement. It is recognized [203] that flavins are somewhat specialized reductants, both biosynthetically and functionally more specific than the much simpler nicotinamide cofactors, which plausibly preceded them [182].

#### 6. *Purine-derived cofactors selected before RNA itself, as opposed to having descended from an RNA world defined through base pairing?*

The overlap between RNA and cofactor biosynthesis, and the incorporation of AMP in several cofactors (where it serves primarily as a “handle” for docking), has been noticed and given the interpretation that cofactors are a degenerated relic of an *oligomer* RNA world [171]. The only significant logical motivation to place oligomer RNA prior to small-molecule cofactors (which are of comparable complexity to *monomer* RNA) is a premise that the elaboration of biosynthesis required selected catalysts, and that RNA base pairing is the least-complex plausible mechanism supporting (specifically, Darwinian) selection and persistence of the required catalysts.

This is a complex premise, as it requires not only organosynthesis of RNA, but also chiral selection and mechanisms to enable base pairing and (presumably template-directed) ligation [204].<sup>45</sup> In comparison, small-molecule catalysis by either RNA [205] or related cofactors may be considered in any context that supports their synthesis.<sup>46</sup> If chemical mechanisms are found which support structured organosynthesis and selection – a requirement for any metabolism-first theory of the origin of life – the default premise may favor simplicity: that heterocycles were first selected as cofactors, and that purine RNA, only one among many species maintained by the same generalized reactions, was subsequently selected for chirality, base-pairing, and ligation.

<sup>45</sup> A particular problem for RNA replication is the steric restriction to 3'-5' phosphate esters, over the kinetically favored 2'-5' linkage.

<sup>46</sup> The relative importance of synthesis and selection depends on whether opening access to a space of reactions, or concentrating flux within a few channels in that space, is the primary limit on the emergence of order at each phase in the elaboration of metabolism. Following our earlier arguments about the need for autocatalysis, selection will be essential in some stages, and this remains an important problem for metabolism-first premises [154]. Chemical selection criteria derived from differential growth rate pose no problem in the domain of small-molecule organocatalysis, but the identification of plausible mechanisms to preserve selected differences remains an important area of work. Most mechanisms that do not derive from RNA base pairing involve separation by spatial geometry or material phases, including porous-medium processes akin to invasion percolation [153], or more general proposals for compositional inheritance [206–208], abstracted from models of coacervate chemistry.

<sup>43</sup> For some reactions, the abstraction of enzyme mechanism is advanced enough to identify small-molecule organocatalysts that could have provided similar functions [199, 200].

<sup>44</sup> Whether the first RNA were produced in this way, or through structurally very dissimilar stages, is a currently active question [201].



### C. The alkyl-thiol cofactors

The major chemicals in this class include the sulfonated alkane-thiols coenzyme-B (CoB) and coenzyme-M (CoM), cysteine and homocysteine including the activated forms S-adenosyl-homocysteine (which under methylation becomes SAM), lipoic acid, and pantetheine or pantothenic acid, including pantetheine-phosphate. The common structure of the alkyl-thiol cofactors is an alkane chain terminated by one or more sulfhydryl (SH) groups. In all cases except lipoic acid, a single SH is bound to the terminal carbon; in lipoic acid two SH groups are bound at sub-adjacent carbons. Differences among the alkyl-thiol cofactors arise from their biosynthetic context, the length of their alkane chains, and perhaps foremost the functional groups that terminate the other ends of the chains. These may be as simple as sulfones (in CoB) or as complex as peptide bonds (in CoA).

Cofactors in this class serve three primary functions, as reductants (cysteine, CoB, pantetheine, and one sulfur on lipoic acid), carriers of methyl groups (CoM, SAM, one sulfur on lipoic acid), and carriers of larger functional groups such as acyl groups (lipoic acid in lipoyl protein, phosphopantetheine in acyl-carrier protein). A highly specialized role in which H is a leaving group is the formation of thioesters at carboxyl groups (pantetheinic acid in CoA, lipoic acid in lipoyl protein). This function is essential to substrate-level phosphorylation [209], and appears repeatedly in the deepest and putatively oldest reactions in core metabolism. A final function closely related to reduction is the formation and cleavage of S – S linkages by cysteine in response to redox state, which is a major controller of both committed and plastic tertiary structure in proteins. The sulfur atoms on cysteine often form coordinate bonds to metals in metallo-enzymes, a function that we may associate with protein ligands, in contrast to the more common nitrogen atoms that coordinate metals in pyrrole cofactors.

The properties of the alkyl-thiol cofactors derive largely from the properties of sulfur, which is a “soft” period-3 element [210] that forms relatively unstable (usually termed “high-energy”) bonds with the hard period-2 element carbon. For the alkyl-thiol cofactors in which sulfur plays direct chemical roles, three main bonds dictate their chemistry: S – C, S – S, and S – H. Sulfur can also exist in a wide range of oxidation states, and for this reason often plays an important role in energy metabolism [211], particularly for chemotrophs, and due to its versatility has been suggested to precede oxygen in photosynthesis [212]. The electronic versatility of sulfur and the high-energy C – S bonds combine with the large atomic radius of sulfur to give access to additional geometrical, electronic and ring-straining possibilities not available to CHON chemistry.

Although not alkyl-thiol compounds as categorized above, two additional cofactors that make important indirect use of sulfur are thiamin and biotin. In neither case is sulfur the element to which transferred C<sub>1</sub> groups are

bound, but its importance to the focal carbon or nitrogen atom is suggested by the complexity of the chemistry and enzymes involved in its incorporation into these two cofactors [166, 213].

#### 1. Biochemical roles and phylogenetic distribution

**Transfer of methyl or methylene groups:** The S atoms of CoM, lipoic acid, and S-adenosyl-homocysteine accept methyl or methylene groups from the nitrogen atoms of pterins. Considering that transition-metal sulfide minerals are the favored substrates for prebiotic direct-C<sub>1</sub> reduction [56, 119, 197], a question of particular interest is how, in mineral scenarios for the emergence of carbon fixation, the distinctive relation between tuned nitrogen atoms in pterins as carbon carriers, and alkyl-thiol compounds as carbon acceptors, would have formed.

**Reductants and co-reductants:** CoB and CoM act together as methyl carrier and reductant to form methane in methanogenesis.<sup>47</sup> A similar role as methylene carrier and reductant is performed by the two SH groups in lipoic acid. CoM is specific to methanogenic archaea [215], while lipoic acid and S-adenosyl-homocysteine are found in all three domains [41, 216]. Lipoic acid is formed from octanoyl-CoA, emerging from the biotin-dependent malonate pathway to fatty acid synthesis, and along with fatty acid synthesis [85], may have been present in the universal common ancestor. The universal distribution of the glycine cycle supports this as noted earlier.

**Role in the reversal of citric-acid cycling:** Lipoic acid becomes the electron acceptor in the oxidative decarboxylation of  $\alpha$ -ketoglutarate and pyruvate in the oxidative Krebs cycle, replacing the role taken by reduced ferredoxin in the rTCA cycle. Thus the prior availability of lipoic acid was an enabling precondition for reversal of the cycle in response to the rise of oxygen.

**Carriers of acyl groups:** Transport of acyl groups in the acyl-carrier protein (ACP) proceeds through thioesterification with pantetheine phosphate, similar to the thioesterification in fixation pathways. In fatty acid

<sup>47</sup> In this complex transfer [120], the fully-reduced (Ni<sup>+</sup>) state of the Nickel tetrapyrrole F<sub>430</sub> forms a dative bond to –CH<sub>3</sub> displacing the CoM carrier, effectively re-oxidizing F<sub>430</sub> to Ni<sup>3+</sup>. Reduced F<sub>430</sub> is regenerated through two sequential single-electron transfers. The first, from CoM-SH, generates a Ni<sup>2+</sup> state that releases methane, while forming a radical CoB’ –S – CoM intermediate with CoB. The radical then donates the second electron, restoring Ni<sup>+</sup>. The strongly oxidizing heterodisulfide CoB-S-S-CoM is subsequently reduced with two NADH in a process known as *electron bifurcation* [214] (described further below), regenerating CoM-SH and CoB-SH while jointly generating the low-potential reductant reduced-ferredoxin. Both the stepwise reduction of F<sub>430</sub> and electron bifurcation illustrate the central role of metals as mediators of single-electron transfer processes in metabolism.

biosynthesis acyl groups are further processed while attached to the pantotheine phosphate prosthetic group.

**Electron bifurcation:** The heterodisulfide bond of CoB-S-S-CoM has a high midpoint potential ( $E'_0 = -140\text{mV}$ ), relative to the  $H^+/H_2$  couple ( $E'_0 = -414\text{mV}$ ), and its reduction is the source of free energy for the *endergonic* production of reduced Ferredoxin ( $\text{Fd}^{2-}$ ,  $E'_0$  *in situ* unknown but between  $-520\text{mV}$  and  $-414\text{mV}$ ) [214], which in turn powers the initial uptake of  $\text{CO}_2$  on  $\text{H}_4\text{MPT}$  in methanogens. The remarkable direct coupling of exergonic and endergonic redox reactions through splitting of binding pairs into pairs of radicals, which are then directed to paired high-potential/low-potential acceptors, is known as *electron bifurcation* [111]. Variant forms of bifurcation are coming to be recognized as a widely-used strategy of metal-center enzymes, either consuming oxidants as energy sources to generate uniquely biotic low-potential reductants such as  $\text{Fd}^{2-}$  [214, 217–219], or to “titrate” redox potential to minimize dissipation and achieve reversibility of redox reactions involving reductants at diverse potentials, *e.g.* by combining low-potential ( $\text{Fd}^{2-}$ ,  $E'_0 = -420\text{mV}$ ) and high-potential (NADH,  $E'_0 = -300\text{mV}$ ) reductants to produce intermediate-potential reductants (NADPH,  $E'_0 = -360\text{mV}$ ) [220]. Together with substrate-level phosphorylation (SLP), electron bifurcation may be the principle chemical mechanism (contrasted with membrane-mediated oxidative phosphorylation) for interconverting biological energy currencies, and along with SLP [209], a mechanism of central importance in the origin of metabolism [221]. Small metabolites including such heterodisulfides of cofactors, which can form radical intermediates exchanging single electrons with Fe-S clusters (typically *via* flavins) are essential sources and repositories of free energy in pathways using bifurcation.

## 2. Participation in carbon fixation pathway modules

The similarity between the glycine cycle and methanogenesis in Fig. 4 emphasizes the convergent roles of alkyl-thiol cofactors. In the glycine cycle, methylene groups are accepted by the terminal sulfur on lipoic acid, and the subadjacent SH serves as reductant when glycine is produced, leaving a disulfide bond in lipoic acid. The disulfide bond is subsequently reduced with NADH. In methanogenesis, a methyl group from  $\text{H}_4\text{MPT}$  is transferred to CoM, with the subsequent transfer to  $\text{F}_{430}$ , and the release from  $\text{F}_{430}$  as methane in the methyl-CoM reductase, coupled to formation of CoB-S-S-CoM. The heterodisulfide is again reduced with NADH, but employs a pair of electron bifurcations to retain the excess free energy in the production of  $\text{Fd}^{2-}$  rather than dissipating it as heat [214]. Methanogenesis is thus associated with 7 distinctive cofactors beyond even the set known to have diversified functions within the archaea [5], again suggesting the derived and highly optimized nature of this Euryarchaeal phenotype. The striking similarity of

these two methyl-transfer systems, mediated by independently evolved and structurally quite different cofactors, suggests evolutionary convergence driven specifically by properties of alkyl thiols.

A curious pattern, which we note but do not attempt to interpret, is the association of non-sulfur, nitrogen-heterocycle cofactors with WL carbon fixation, contrasted with the use of sulfur-containing heterocycles in carboxylation reactions of the rTCA cycle. The non-sulfur cofactors THF and  $\text{H}_4\text{MPT}$  are used in the reactions of the WL pathway, while the biosynthetically-related but sulfur-containing cofactor Thiamin mediates the carbonyl insertion (at a thioester) in rTCA [84, 222]. Biotin – which has been generally associated with malonate synthesis in the fatty-acid pathway (and derivatives such as propionate carboxylation to methyl-malonate in 3HP [85]) – mediates the subsequent  $\beta$ -carboxylation of pyruvate and of  $\alpha$ -ketoglutarate [86, 223, 224]. Thus the two cofactors we have identified as using sulfur indirectly to tune properties of carbon or nitrogen  $\text{C}_1$ -bonding atoms mediate the two chemically quite different sequential carboxylations in rTCA.

## D. Carboxylation reactions in cofactor synthesis

Carboxylation reactions can be classified as falling into two general categories: those used in core carbon “uptake”, and those used exclusively in the synthesis of specific cofactors. In addition to carboxylation reactions in carbon-fixation pathways, the former category includes the carboxylation of crotonyl-CoA in the glyoxylate regeneration cycle. Although not an autotrophic pathway this reaction does form a distinct entry point for  $\text{CO}_2$  into the biosphere. The carboxylation of acetyl-CoA to malonyl-CoA further serves a dual purpose, in being both the starting point for fatty acid synthesis, as well as a key step in the 3HP pathway used in several carbon-fixation pathways. All these carboxylation reactions thus have in common that they are used at least in some organism as the central source for cellular carbon. All other carboxylation reactions that are not used as part of core carbon uptake, are used in the synthesis of the biotin cofactor, and the purine and pyrimidine nucleotides (see Fig. 17).

If we consider the sequences in which  $\text{CO}_2$  is incorporated in these pathways, they also form a distinct class of chemistry. In all three cases the resulting carboxyl group is immediately aminated, either as part of the carboxylation reaction, or in the following reaction, and the carboxamide group is subsequently maintained into the final heterocyclic structure. In addition we previously saw that IMP becomes the source for the folate and flavin family (through GTP). Carboxylation reactions are thus either a general source for cellular carbon in core metabolism, or a specific source of carboxamide groups in the synthesis of cofactors that are part of the catalytic control of core metabolism.

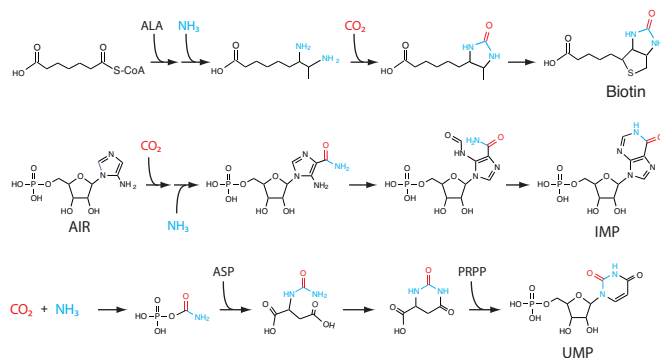


FIG. 17: Carboxylation reactions in the synthesis of cofactors. The sequences show the immediate amination of the carboxyl group to a carboxamide group, which is then preserved into the final heterocyclic structure. As the only carboxylations not used in core carbon uptake, these reaction sequences form a distinct class of chemistry. Amination reactions are shown as net additions of ammonia, which may be derived from other sources (such as glutamine, aspartate or S-adenosyl-methionine). Abbreviations: Alanine (ALA); Aspartate (ASP); phosphoribosyl pyrophosphate (PRPP).

### E. The chorismate pathway in both amino acid and cofactor synthesis

Chorismate is the sole source of single benzene rings in biochemistry [225]. The non-local  $\pi$ -bond resonance is used in a variety of charge-transfer and electron transfer and storage functions, in functional groups and cofactors derived from chorismate. We have noted the charge-transfer function of PABA in tuning  $N^{10}$  of folates, and its impact on  $C_1$  chemistry. The para-oriented carbonyl groups of quinones may be converted to partially- or fully-resonant orbitals in the benzene ring, enabling fully oxidized (quinone), half-reduced (semiquinone), or fully reduced (hydroquinone) states [203]. Finally, the aromatic ring in tryptophan (a second amino acid which behaves in many ways like a cofactor) has at least one function in the active sites of enzymes as a mediator of non-local electron-transfers [226].

## V. INNOVATION: PROMISCUOUS CATALYSIS, SERENDIPITOUS PATHWAYS

The previous sections argued for the existence of low-level chemical and cofactor/catalyst constraints on metabolic innovations, and presented evolutionary divergences that either respected these as constraints, or were enabled by the diversification of cofactor and catalytic functions. In this section we consider the dynamics by which innovation occurs, and its main organizing principles. Innovation in modern metabolism occurs principally by duplication and divergence of enzyme function [116, 227, 228]. Often it relies on similarity of func-

tions among paralogous enzymes, but in some cases may exploit more distant or accidental overlap of functions.

Innovation always requires some degree of enzymatic promiscuity [116], which may be the ability to catalyze more than one reaction (catalytic promiscuity) or to admit more than one substrate (substrate ambiguity). Pathway innovation also requires *serendipity* [229], which refers to the co-incidence of new enzymatic function with some avenue for pathway completion that generates an advantageous phenotype from the new reaction. Most modern enzymes are highly specific,<sup>48</sup> but specific enzymes – whether due to structure or due to evolved regulation – are of necessity diversified in order to cover the broad range of metabolic reactions used in the modern biosphere. Serendipitous pathways assembled from a diversified inventory of specific enzymes will in most cases be strongly historically contingent as they depend on either overlap of narrow affinity domains or on “accidental” enzyme features not under selection from pre-existing functions. Such pathways therefore seem unpredictable from first principles; whether they are rare will depend on the degree to which the diversity of enzyme substrate-affinities compensates for their specificity.

A key question for early metabolic evolution is whether the trade-off between specificity and diversity was different in the deep past than in the present, in either degree or in structure, in ways that affected either the discovery of pathway completions or the likelihood that new metabolites could be retained within existing networks. These structural aspects of promiscuity and serendipity determine the regulatory problem faced by evolution in balancing the elaboration of metabolism with its preservation and selection for function.

### 1. Creating reaction mechanisms and restricting substrates, while evolving genes

Metabolism is characterized at all levels by a tension between creating reaction mechanisms that introduce new chemical possibilities, and then pruning those possibilities by selectively restricting reaction substrates. Whether this tension creates a difficult or an easy problem for natural selection to solve depends at any time on whether the accessible changes in catalytic function, starting from integrated pathways, readily produce new integrated pathways whose metabolites can be recycled in autocatalytic loops. We argue that the conservation of pathway mechanisms, particularly when these are defined by generic functional groups such as carboxyls, ketones,

<sup>48</sup> However, broad substrate-specificity is no longer considered rare, and is even explained as an expected outcome in some cases where costs of refinement are higher than can be supported by natural selection, and in other cases by positive selection for phenotypic plasticity [228]

and enols, with promiscuity coming from substrate ambiguity with respect to molecular properties away from the reacting functional group, favors the kind of orderly pathway duplication that we observe in the extant diversity of core metabolism. Therefore we expect that serendipitous pathway formation was both facile in those instances in the early phases of metabolic evolution where innovations in radical-based mechanisms for carbon incorporation occurred, and structured according to the same local-group chemistry around which the substrate network is organized [67].

Modern enzymes both create reaction mechanisms and restrict substrates, but the parts of their sequence and structure that are under selection for these two categories of function may be quite different, so the two functions can evolve to a considerable degree independently. Active-site mechanisms in enzymes for organic reactions will often depend sensitively on a small number of highly conserved catalytic residues in a relatively fixed geometry, while substrate selection can depend on a wide range of properties of enzyme shape or conformation dynamics [228], on local functional-group properties of the substrate that have been termed “chemophores” [230], as well as (in some cases) on detailed relations between the substrate and active-site geometry or residues. An extreme example of the potential for separability between reaction mechanism and substrate selection is found in the polymerases. A stereotypical reaction mechanism of attack on activating phosphoryl groups requires little more than correct positioning of the substrates. In the case of DNA polymerases, at least six known categories (A, B, C, D, X, and Y) with apparently independent sequence origin have converged on a geometry likened to a “right hand” which provides the required orientation [231, 232].

At the same time as evolving enzymes needed to provide solutions to the biosynthetic problem of enabling and regulating metabolic network expansion, they were themselves dependent on the evolving capabilities of genomic and translation systems for maintaining complexity and diversity. Jensen [227] originally argued that high enzymatic specificity was no more plausible in primitive cells than highly diversified functionality,<sup>49</sup> and that enzymatic promiscuity was both evolutionarily necessary and consistent with what was currently known about

substrate ambiguity and catalytic promiscuity. Modern reviews [116, 228, 230] of the mechanisms underlying functional diversity, promiscuity, and serendipity confirm that substrate ambiguity is the primary source of promiscuity that has led to the diversification of enzyme families. It is striking that, even in cases where substrate affinity has been the conserved property while alternate reaction mechanisms or even alternate active sites have been exploited, it is often local functional groups on one or more substrates that appear to determine much of this affinity [228].

## 2. Evidence in our module substructure that early innovation was governed principally by local chemistry

The substructure of modules, and the sequence of innovations, we have sketched in Sec. III appears to be dominated by substrate ambiguity in enzymes or enzyme families with conserved reaction mechanisms. The key reactions in carbon fixation are of two types: Crucial reactions typically involve metal centers or cofactors that could have antedated enzymes, and it is primarily reaction sites, not molecular selectivity, that distinguishes pathways at the stage of these reactions<sup>50</sup>. The shared internal sequence of reductions and isomerizations common to modules (Fig. 8) are very broadly duplicated, and the molecular specificity in their enzymes today is not correlated with significant reaction-sequence changes in the internal structure of pathways. These pathways could plausibly function much as they do today with less-specific hydrogenases and aconitases.

A quantitative reconstruction of early evolutionary *dynamics* will require merging probability models for networks and metabolic phenotypes with those for sequences and structure of enzyme families. The goal is a consistent model of the temporal sequence of ancestral states of catalyst families, and of the substrate networks on which they acted.

## VI. INTEGRATION OF CELLULAR SYSTEMS

The features of metabolism that display a “logic” of composition, which is then reflected in their evolutionary history, are those with few and robust responses to environmental conditions that can be inferred from present diversity. These are the subsystems whose evolution has been simplified and decoupled by modularity. Their relative immunity from historical contingency, resulting in more “thermodynamic” modes of evolution, result from

<sup>49</sup> This argument was largely a rebuttal of an earlier proposal by Horowitz [233] for “retrograde evolution” of enzyme functions. The 1940s witnessed the rise of an overly-narrow interpretation of “one gene, one enzyme, one substrate, one reaction” (a rigid codification of what would become Crick’s *Central Dogma* [234], which in the context of complex pathway evolution appeared to be incompatible with natural selection for function of intermediate states. The Horowitz solution was to depend on an all-inclusive “primordial soup” [50], in which pathways could grow backward from their final products, propagating selection step-wise downward in the pathway until a pre-existing metabolite or inorganic input was found as a pathway origin.)

<sup>50</sup> Recall that the enzymes that have been argued to be the ancestral forms of both the acetyl- and succinyl-CoA ligases and the pyruvate and  $\alpha$ -ketoglutarate biotin-carboxylases show very close sequence homology [86, 89], suggesting shared ancestral enzymes for both.

rapid, high-probability convergence in populations that can share innovations [141].

The larger roles for standing variation and historical contingency that are so often emphasized [235] in accounts of evolutionary dynamics are made possible by longer-range correlations that link modules, creating mutual dependencies and restricting viable changes [108, 109]. The most important source of such linkage in extant life is the unification of metabolic substrates and control processes within cells [236]. Cellular death or reproduction couples fitness contributions from many metabolic-phenotype traits, together with genome replication systems. This enables the accumulation of diversity as genomes capture and exploit gains from metabolic control, complementary specialization [237], and the emergence of ecological assemblies of specialists as significant mediators of contingent aspects of evolutionary innovation (as we illustrated with the example of methylotrophy).

We consider in this section several important ways in which aggregation of metabolic processes within cells follows its own orderly hierarchy and progression. We note that even a single cell does not impose only one type of aggregation, but at least three types, and that these are the bases for different selection pressures and could have arisen at different times. Within cellular subsystems, the coupling of chemical processes is often mediated by coupling of their energy systems, which has probably developed in stages we can identify. Finally, even where molecular replication is coupled to cellular physiology, in the genetic code, strong and perhaps surprising signatures of metabolic modularity are recapitulated.

#### A. Cells provide at least three functionally distinct forms of compartmentation

Under even the coarsest functional abstraction, the cell provides not one form of compartmentation, but at least three [238, 239]. The geometry and topology of closed spheres or shells, and the capacitance and proton impermeability of lipid bilayers, permit the buildup of pH and voltage differences, and thus the coupling of redox and phosphate energy systems through intermediate proton-motive (or in many cases, sodium-motive) force [240]. The concentration of catalysts with substrates enhances reactions that are second-order in organic species, and the equally important homeostatic control of the cytoplasm regulates metabolic reaction rates and precludes parasitic reactions. Finally, the cell couples genetic variations to internal biochemical and physiological variations<sup>51</sup> much more exclusively than they are coupled to

shared resources such as biofilms or siderophores, leading to the different evolutionary dynamics of development from niche construction [38].<sup>52</sup> Each of these forms of coupling affects the function and evolution of the modules we have discussed.

##### 1. *Coupling of redox and phosphate energy systems may have been the first form of compartmentation selected*

Biochemical subsystems driven, respectively, by redox potential or phosphoanhydride-bond dehydration potential, cannot usually be directly coupled to one another due to lack of “transducer” reactions that draw on both energy systems.<sup>53</sup> The notable exception to this rule is the exchange of phosphate and sulfur groups in substrate-level phosphorylation [203] from thioesters (which may proceed in either direction depending on conditions). Although it provides a less flexible mode of coupling than membrane-mediated oxidative phosphorylation, this crucial reaction type, which occurs in some of the deepest reactions in biochemistry (those employing CoA, including all those in the six carbon fixation pathways), has been proposed as the earliest coupling of redox and phosphate [209], and the original source of phosphoanhydride potential [69] enabling pathways that require both reduction and dehydration reactions.

Phosphate concentration limits growth of many biological systems today, and phosphate concentrations appear to be even lower in vent fluids [244] than on average in the ocean, making it difficult to account for the emergence of many metabolic steps in hydrothermal vent scenarios for the origin of life. Serpentinization and other rock-water interactions that produce copious reductants also scavenge phosphates into mineral form, so it appears doubtful that phosphates were abundant in the environments otherwise most favorable to geochemical organosynthesis. What little phosphate is found in water is primarily orthophosphate, because the phosphoanhydride bond is unstable to hydrolysis. Therefore the retention of orthophosphate, and the continuous regeneration of pyrophosphate and polyphosphates [245–249], may have been essential to the spread of early life beyond very rare geochemical environments.

The membrane-bound ATP-synthetase, which couples phosphorylation to a variety of redox reactions [5]

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the complex ecosystems including viral RNA and DNA that are partly autonomous of the physiology of particular cells [241, 242].

<sup>52</sup> For an argument that somatic development and niche construction are variants on a common process, distinguished by the genome’s level of control and exploitation of the constructed resources, see Ref. [243].

<sup>53</sup> In addition to the ultimate physical constraint of limits to free energy, biochemistry operates under additional proximate constraints not only from availability of free energy but from the chemical and quantum-mechanical substrates in which it is carried.

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<sup>51</sup> The perspective that this is an active coupling, which defines one of the forms of *individuality* rather than providing a complete characterization of the nature of the living state, is supported by

through proton or sodium pumping, is therefore essential in nearly all biosynthetic pathways, and must have been among the first functions of the integrated cell. Without a steady source of phosphate esters, none of the three oligomer families could exist. The ATP synthetase itself is homologous in all organisms, providing one strong argument (among many [85, 250]) for a membrane-bound last common ancestor. Proton-mediated phosphorylation (best known through oxidative phosphorylation in the respiratory chain [203]) requires a topologically enclosed space to function as a proton capacitor [240]. However, as shown by gram-negative bacteria [5] and their descendants mitochondria and plastids, which acidify the periplasmic space or thylakoid lumen, the proton capacitor need not be (and generally is not) the same compartment as the cytoplasm containing enzymatic reactions. Because the coupling of energy systems is a different function from regulating reaction rates catalytically, the phosphorylation system should not generally have been subject to the same set of evolutionary pressures and constraints as other cellular compartments, and need not have arisen at the same time. We note that, because it may have lower osmotic pressure than the cytoplasm, the acidified space required for proton-driven phosphorylation may not have required a cell wall, greatly simplifying the number of concurrent innovations required for compartmentalization, compared to those for the cytoplasm. Therefore we conjecture that proton-mediated phosphorylation could have been the first function leading to selection for lipid-bilayer compartmentalization, allowing other cellular functions to accrete at later times.

*2. Regulation of biosynthetic rates may have been prerequisite for the optimization of loop-autocatalytic cycles*

The second function of cellular compartments, and the one most emphasized in vesicle theories of the origin of life [6, 251, 252], is the enhancement of second-order reactions by collocation of catalysts and their substrates. Here we note another role that we have not seen mentioned, which is more closely related to the functions of the cell that *inhibit* reactions. Organisms employing autocatalytic-loop carbon fixation pathways must reliably limit their anabolic rates to avoid drawing off excess network catalysts into anabolism, resulting in passage below the autocatalytic threshold for self-maintenance, and collapse of carbon fixation and metabolism. Regulating anabolism to maintain viability and growth may have been an early function of cells.

We noted in Sec. IIID 4 the fragility of autocatalytic-loop pathways to parasitic side-reactions, and the way the addition of a linear pathway such as WL stabilizes loop autocatalysis in the root node of Fig. 11.<sup>54</sup> It may

be that the optimizations in either branch of the carbon-fixation tree were not possible until rates of anabolism were sufficiently well-regulated to protect supplies of loop intermediates or essential cofactors. Therefore, while the root node is plausible as a pre-cellular [56] or an early cellular (but non-optimized) form, either branch from it may have required the greater control afforded by quite refined cellular regulation of reaction rates.

**B. Coupling of metabolism to molecular replication, and signatures of chemical regularity in the genetic code**

Among the subsystems coupled by modern cells, perhaps none is more elaborate than the combined apparatus of amino acid and nucleotide biosynthesis and protein coding. The most remarkable chemical aspect of the protein-coding system is that it is an *informational* system: a sophisticated machinery of transcription, tRNA formation and aminoacylation, and ribosomal translation separates the chemical properties of DNA and RNA from those of proteins, permitting almost free selection of sequences in both alphabets in response to requirements of heredity and protein function.<sup>55</sup> The interface at which this separation occurs is the genetic code. From the informational suppression of chemical details that defines the coding system, the code itself might have been expected to be a random map, but empirically the code is known to contain many very strong regularities related to amino acid biosynthesis and chemical properties, and perhaps to the evolutionary history of the aminoacyl-tRNA synthetases.

Many explanations have been advanced for redundancy in the genetic code, as a source of robustness of protein properties against single-point mutations [141, 253–255], but in all of these the source of selection originates in the elaborate and highly evolved function of coding itself. In many cases the redundancy of amino acids at adjacent coding positions reflects chemical or structural similarities, consistent with this robustness-criterion for selection, but in nearly all cases redundancy of bases in the code correlates even more strongly with shared elements of *biosynthetic pathways* for the amino acids. The co-evolutionary hypothesis of Wong [256] accounts for the correlation of the first base-position with amino-acid backbones as a consequence of duplication and diver-

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be hazardous, if catalysts in the main fixation pathway to not sufficiently accelerate their reaction rates, creating a separation of timescales relative to the uncatalyzed background. Within the first cells, the same hazard is posed by secondary anabolism, as its reaction rates become enhanced by catalysts similar to those in the core. This fact was clearly noted already in Ref. [56].

<sup>55</sup> The observation that enzymes acting on DNA have evolved to actively mitigate chemical differences in the bases, to enable a more nearly neutral combinatorial alphabet, is due to Peter Schuster (pers. comm.)

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<sup>54</sup> For proto-metabolism, spontaneous abiotic side-reactions may



gence of amino acid biosynthetic enzymes together with aminoacyl-tRNA synthetases (aaRS). The stereochemical hypothesis of Woese [257] addresses a correlation of the second coding position with a measure of hydrophobicity called the *polar requirement*. The remarkable fact that both correlations are highly significant relative to random assignments, but that they are *segregated* between first and second codon bases, is not specifically addressed in either of these accounts. Copley *et al.* [205] address the same regularities as both the Wong and Woese hypotheses, but link them to much more striking redundancies in biosynthetic pathways, which they propose are consequences of small-molecule organo-catalytic roles of dimer RNA in the earliest biosynthesis of amino acids.

We note here a further chemical regularity in the genetic code, which falls outside the scope of the previous explanations: purines in second base-position code for several amino acids that either use related purine-derived cofactors in their biosynthetic pathways, or are directly related to the codon. This association is much more comprehensive for G-second codons than for A-second codons, and it does not suggest the same kinds of mechanistic relations in the two cases. However, it further compresses the description of patterns in the code that were not addressed in Ref. [205], in terms of similar chemical and biosynthetic associations.

A strong correlation, of a single kind, is found between the glycine cycle for amino acid biosynthesis from C<sub>1</sub> groups on folate cofactors, and codons XGX, where X is any base and G is guanine. (In what follows we abbreviate wobble-base positions y for pyrimidines U and C, or u for purines A or G.) This group includes glycine (GGX), serine (AGy), cysteine (UGy), and tryptophan (UGu).<sup>56</sup> We do not propose a specific mechanism for such an association here, but our earlier argument that folates would have been contemporaneous with GTP suggests that biosynthesis through the glycine cycle was the important source of these amino acids at the time they became incorporated into the code. Some of these amino acids satisfy multiple regularities, as in the correlation of glycine with GXX ↔ reductive transamination, or cysteine with UXX ↔ pyruvate backbone, proposed in Ref. [205].

The position (CAy) of histidine, synthesized from ATP, is the only case we recognize of a related correlation in XAX codons. For this position, the availability of ATP seems to have been associated with the synthesis of histidine directly through the cyclohydrolase function (rather than through secondary cofactor functions), at the time this amino acid became incorporated into the code.

Much more than correlation is required to impute causation, and all existing theories of cause for regularities

in the genetic code are either highly circumstantial or require additional experimental support. Therefore we limit the aspects of these observations that we regard as significant to the following three points:

**The existence of a compression:** The idealized adaptive function of coding is to give maximum evolutionary plasticity to aspects of phenotype derived from protein sequence, uncoupled from constraints of underlying biosynthesis. The near-wholesale transition from organic chemistry to polymer chemistry around the C<sub>20</sub> scale suggests that this separation has been effectively maintained by evolution. Strong regularities which make the description of the genetic code *compressible* relative to a random code reflect *failures* of this separation which have transmitted selection pressure across levels, during either the emergence or maintenance of the code. These include base-substitution errors, whether from mutation or in the transcription and translation processes, but also apparently chemical relations between nucleobases and amino acids.

**The segregation of the roles of different base positions and in some cases different bases in terms of their biochemical correlates:** The genetic code is like a “rule book” for steps in the biosynthesis of many amino acids,<sup>57</sup> but the chemical correlations which are its rules are of many kinds. Beyond the mere existence of those rules, and their collective role as indices of regularity threading the code, we must explain why rules of different kinds are so neatly *segregated* over different base positions and sometimes over different bases (as in the XGX and XAX codons).

**A compression that references process rather than property:** The role of biosynthetic pathways as correlates of regularities makes this compression of the genetic code a reference to the *process* and metabolic network context within which amino acids are produced, and not merely to their *properties*. (Many of the chemical properties recognized as criteria of selection, whether size or hydrophobicity, are shared at least in part because they result from shared substrates or biosynthetic steps.) We think of the function of coding as separating biosynthetic process from phenotype: transcription and translation are “Markovian” in the sense that the only information from the biosynthetic process which survives to affect the translated protein is what is inherent in the structure of the amino acid.<sup>58</sup> Thus selection on post-translation phenotypes should only be responsive to the finished amino acids. The existence of regularities in the genetic code which show additional correlation with

<sup>56</sup> Both purines are used in the mitochondrial code and only UGG in the nuclear code.

<sup>57</sup> The correlations in the code may be understood as rules because the biosynthetic pathways may be placed on a decision tree, with branches labeling alternative reactions at several stages of synthesis, and branching directions indicated by the position-dependent codon bases [205].

<sup>58</sup> In technical terms, one says that the phenotype is *conditionally independent* of the biosynthetic pathway, *given* the amino acid.

intermediate steps in the biosynthetic process therefore requires either causes other than selection on the post-coding phenotype (including its robustness), or a history-dependence in the formation of the code that reflects earlier selection on intermediate pathway states. If they reflect causal links to metabolic chemistry, these “failures” of the separation between biosynthetic constraint and selection of polymers for phenotype may have broken down the emergence of molecular replication into a sequence of simpler, more constrained, and therefore more attainable steps.

## VII. CONCLUSIONS

We have argued that the fundamental problem of electron transfer in aqueous solution leads to a qualitative division between catalytically “hard” and “easy” chemistry, and that this division in one form or another has led to much of the architecture and long-term evolution of the biosphere. Hard chemistry involves electron transfers whose intermediate states would be unstable or energetically inaccessible in water if not mediated by transition-metal centers in metal-ligand complexes. Easy chemistry involves hydrogenations and hydrations, intramolecular redox reactions, and a wide array of acid-base chemistry. Easy chemistry is promiscuously re-used and provides the internal reactions within modules of core metabolism. Hard chemistry defines the module boundaries and the key constraints on evolutionary innovation. These simple ideas underlie a modular decomposition of carbon fixation that accounts for all known diversity, largely in terms of unique adaptations to chemically simple variations in the abiotic environment. On the foundation of core metabolism laid by carbon fixation, the remainder of biosynthesis is arranged as a fan of increasingly independent anabolic pathways. The unifying role of the core permits diverse anabolic pathways to independently reverse and become catabolic, and the combinatorics of possible reversals in communities of organisms determines the space of evolutionary possibilities for heterotrophic ecology.

We have emphasized the role of feedback in biochemistry, which takes different forms at several levels. Network autocatalysis, if we take as a separate question the origin of external catalytic and cofactor functions, is found as a property internal to the small-molecule substrate networks for many core pathways. A qualitatively different form of feedback is achieved through cofactors, which may act either as molecular or as network catalysts. As network catalysts they differ from small metabolites because their internal structure is not changed except at one or two bonds, over the reactions they enable. The cofactors act as “keys” that incorporate domains of organic chemistry within biochemistry, and this has made them both extraordinarily productive and severely limiting. No extant core pathways function without cofactors, and cofactor diversification ap-

pears to have been as fundamental as enzyme diversification in some deep evolutionary branches. We have therefore argued for a closely linked co-evolution of cofactor functions with the expansion of the universal metabolic network from inorganic inputs, and attempted to place key cofactor groups within the dependency hierarchy of biosynthetic pathways, particularly in relation to the first ability to synthesize RNA.

The most important message we hope to convey is the remarkable imprint left by very low-level chemical constraints, even up to very high levels of biological organization. Only seven carbon fixation modules, mostly determined by distinctive, metal-dependent carboxylation reactions, cover all known phylogenetic diversity and provide the building blocks for both autotrophic and heterotrophic metabolic innovation. A similar, small collection of organic or organometallic cofactor families have been the gateways that determine metabolic network structure from the earliest cells to the present. The number of these cofactors that we consider distinct may be somewhat further reduced if we recognize biosynthetic relatedness that leads to functional relatedness (as in the purine-derived or chorismate-derived cofactors), or cases of evolutionary convergence dominated by properties of elements (as for lipoic acid and the CoB-CoM system).

We believe that these regularities should be understood as laws of biological organization. In a proper, geochemically-embedded theory of the emergence of metabolism, they should be predictable, either particular forms as in the case of metal chemistry or convergent uses of nitrogen and sulfur, or properties of distributions as in the use of network modules or the diversity of cofactors. Moreover, this lawfulness should have been expected: the factors that reduce (or encrypt) the role of laws in biology, and lead to unpredictable historical contingencies, arise from long-range correlation. Correlation of multiple variables leads to large spaces of possibility and entangles the histories of different traits, making the space difficult to sample uniformly. But correlation in biology is in large part a *constructed* property; it has not been equally strong in all eras and its persistence depends on timescales. Long-term evolution permits recombination even in modern integrated cells and genomes. Early life, in contrast, with its less-integrated cells and genomes, and its more loosely-coupled traits, had constructed less long-range correlation. These are the domains where the simpler but invariant constraints of underlying chemistry and physics should show through.

## Acknowledgments

This work was completed as part of the NSF FIBR grant nr. 0526747: From geochemistry to the genetic code. DES thanks Insight Venture Partners for support. RB is further supported by an Omidyar Fellowship at the Santa Fe Institute. We are grateful to Harold Morowitz, Shelley Copley, and Charles McHenry for critical

conversations of these ideas and essential references.

## Appendix A: Bipartite graph representations for chemical reaction networks

The stoichiometry of a chemical reaction may be represented by a *directed hypergraph* [258]. A hypergraph differs from a simple graph in that, where each edge of a simple graph has two points as its boundary, in a hypergraph, a hyper-edge may have a set of points as its boundary. In a directed hypergraph, the input and output sets in the boundary are distinguished. For the application to chemistry presented here, each hyper-edge corresponds to a reaction, and its input and output boundary sets correspond to moles of the reactant and product molecules.

It is possible to display the hypergraphs representing chemical reactions as *doubly-bipartite* simple graphs, meaning that both nodes and edges exist in two types, and that well-formed graphs permit only certain kinds of connections of nodes to edges. The bipartite graph representation of a reaction has an intuitive similarity to the conventional chemical-reaction notation (shown in Fig. A1 below), but it makes more explicit reference to the chemical mass-action law as well as to the reaction stoichiometry. For appropriately constructed graphs, graph-rewrite rules correspond one-to-one with evaluation steps of mass-action kinetics, permitting simplification of complex reaction networks to isolate key features, while retaining correspondence of the visual and mathematical representations.

We use graph representations of reaction networks in the text where we need to show relations among multiple pathways that may connect the same inputs and outputs (such as acetyl-CoA and succinyl-CoA), and may draw from the same input and output species (such as  $\text{CO}_2$ , reductant, and water). Parallel input and output sequences appear as “ladder” topology in these graphs, and for the particular pathways of biological carbon fixation, this is due to the recurrence of identical functional-group reaction sequences in multiple pathways, as discussed in Sec. III B.

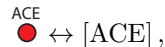
In this appendix we define the graph representation used in the text, introduce graph-reduction procedures and prove that they satisfy the mathematical property of associativity, and provide solutions for the particular simplification of interacting rTCA and Wood-Ljungdahl pathways in a diluting environment.

### 1. Definition of graphic elements

#### a. Basic elements and well-formed graphs

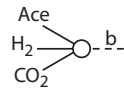
The elements in a bipartite-graph representation of a chemical reaction or reaction network are defined as follows:

- Filled dots represent concentrations of chemical species. Each such dot is given a label indicating the species, such as



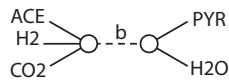
used to refer to acetate in the text.

- Dashed lines represent transition states of reactions. Each is given a label indicating the reaction, as in  $\text{---}b\text{---}$ .
- Hollow circles indicate inputs or outputs between molecular species and transition states, as in



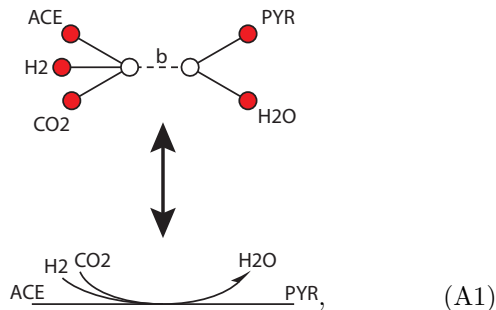
Each circle is associated with the complex of reactants or products to the associated reaction, indicated as labeled line stubs.

- Hollow circles are tied to molecular concentrations with solid lines  $\xrightarrow{\text{ACE}}$ ; one line per mole of reactant or product participating in the reaction. (That is, if  $m$  moles of a species A enter a reaction  $b$ , then  $m$  lines connect the dot corresponding to [A] to the hollow circle leading into reaction  $b$ . This choice uses graph elements to carry information about stoichiometry, as an alternative to labeling input- or output-lines to indicate numbers of moles.)
- Full reactions are defined when two hollow circles are connected by the appropriate transition state, as in



describing the reductive carboxylation of acetate to form pyruvate.

- The bipartite graph for a fully specified reaction takes the form



where labeled stubs are connected to filled circles by mole-lines. The bipartite-graph corresponds to the standard chemical notation for the same reaction as shown.

*b. Assignment of graph elements to terms in the mass-action rate equation*

The mass-action kinetics for a graph such as the reductive carboxylation of acetate<sup>59</sup> is given in terms of two half-reaction currents, which we may denote with the reaction label and an arbitrary sign as

$$\begin{aligned} j_b^+ &= k_b [\text{ACE}] [\text{CO}_2] [\text{H}_2] \\ j_b^- &= \bar{k}_b [\text{PYR}] [\text{H}_2\text{O}]. \end{aligned} \quad (\text{A2})$$

$k_b$  and  $\bar{k}_b$  denote the forward and reverse half-reaction rate constants. The total reaction current  $J_b \equiv j_b^+ - j_b^-$  is related to the contribution of this reaction to the changes in concentration as

$$\begin{aligned} [\dot{\text{ACE}}] &= [\dot{\text{CO}}_2] = [\dot{\text{H}}_2] = -J_b \\ [\dot{\text{PYR}}] &= [\dot{\text{H}}_2\text{O}] = J_b, \end{aligned} \quad (\text{A3})$$

where the overdot denotes the time derivative. Reaction currents on graphs do not have inherent directions, reflecting the microscopic reversibility of reactions. All sources of irreversibility are to be made explicit in the chemical potentials that constitute the boundary conditions for reactions.

Each term in the mass-action rate equation may be identified with a specific graphical element in the bipartite representation. The half-reaction rate constants  $k_b$ ,  $\bar{k}_b$  are associated with the hollow circles, and the current  $J_b$  (which is the time-derivative of the coordinate giving the “extent of the reaction”) is associated with the transition-state dashed line. Concentrations, as noted, are associated with filled dots, and stoichiometric coefficients are associated with the multiplicities of solid lines.

## 2. Graph reduction for reaction networks in steady state

Networks of chemical reactions in steady state satisfy the constraints that the input and output currents to each chemical species (including any external sources or sinks) sum to zero. These constraints are the basis of stoichiometric flux-balance analysis [259–262], but they can also be used to eliminate internal nodes as explicit variables, leading to lumped-parameter expressions for entire sub-networks as “effective” vertices or reactions. With

appropriate absorption of externally buffered reagents into rate constants, this network reduction can be done exactly, without loss of information. An example of such a reduction is the Michaelis-Menton representation of multiple substrate binding at enzymes. Systematic methods for network reduction were one motivation behind Sinanoglu’s graphic methods [263, 264]. More sophisticated stochastic approaches have recently been used to include fluctuation properties in effective vertices, generalizing the Michaelis relation beyond mean field [265].

The map we have given of mass-action rate parameters to graphic elements allows us to represent steady-state network reduction in terms of graph reduction. In this approach, rewrite rules for the removal of graph elements are mapped to composition rules for half-reaction rate constants and stoichiometric coefficients. These composition rules can be proved to be associative, leading to an algebra for graph reduction. Here we sketch the rewrite rules relevant to reduction of the citric-acid cycle graph. In the next subsection we will reduce the graph, to the form used in the text.

*a. The base composition rule for removal of a single internal species*

The simplest reduction is removal of an intermediate chemical species that is the sole output to one reaction, and the sole input to another, in a linear chain. Examples in the TCA cycle include malate (MAL) and isocitrate (ISC), produced by reductions and consumed by dehydrations. They also include citrate (CIT) itself, produced by the hydration of aconitate and consumed by retro-aldol cleavage.

For a single linear reaction as shown in Fig. 18, the mass-action law is

$$[\text{A}] k_a - [\text{B}] \bar{k}_a = J_a, \quad (\text{A4})$$

and concentrations change as

$$\begin{aligned} \dot{[\text{A}]} &= -J_a \\ \dot{[\text{B}]} &= J_a. \end{aligned} \quad (\text{A5})$$

The equilibrium constant for the reaction  $\text{A} \rightarrow \text{B}$  is

$$K_{\text{A} \rightarrow \text{B}} = \frac{k_a}{\bar{k}_a}. \quad (\text{A6})$$

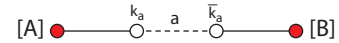


FIG. 18: Basic reaction graph.  $[\text{A}]$  and  $[\text{B}]$  are concentrations associated with the two colored nodes. Forward and backward rate constants  $k_a$  and  $\bar{k}_a$  are associated with the two unfilled circles. The associated reaction state current is  $J_a$ .

<sup>59</sup> All examples in this appendix use the same simplified projection onto the CHO sector that is used in diagrams in the main text. Actual reaction free energies will be driven by coupled energies of hydrolysis of ATP or oxidation of thiols to thioesters. The graph-reduction methods described in the next section may be used to include such effects into lumped-parameter representations of multi-reagent reaction sequences that regenerate energetic intermediates such as ATP or CoA in a network where these are made explicit.

For two such reactions in a chain, as shown in Fig. 19, the mass-action laws are

$$\begin{aligned} [A] k_a - [X] \bar{k}_a &= J_a \\ [X] k_b - [B] \bar{k}_b &= J_b, \end{aligned} \quad (\text{A7})$$

and the conservation laws become

$$\begin{aligned} \dot{[A]} &= -J_a \\ \dot{[X]} &= J_a - J_b \\ \dot{[B]} &= J_b. \end{aligned} \quad (\text{A8})$$

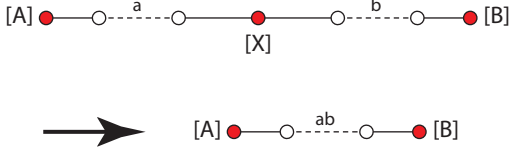


FIG. 19: Removal of an internal species X from a diagram with elementary reactions. Rate constant pairs  $(k_a, \bar{k}_a)$ ,  $(k_b, \bar{k}_b)$  are used to define new rate constants  $(k_{ab}, \bar{k}_{ab})$  for the effective transition state  $ab$ .

Under the steady-state condition  $\dot{[X]} = 0$ , we wish to replace the equations (A7,A8) with a rate equation

$$[A] k_{ab} - [B] \bar{k}_{ab} = J_{ab} \quad (\text{A9})$$

and a conservation law expressed in terms of  $J_a = J_{ab} = J_b$ . The rate constants in Eq. (A9) are to be specified through a composition rule

$$(k_a, \bar{k}_a) \circ (k_b, \bar{k}_b) = (k_{ab}, \bar{k}_{ab}) \quad (\text{A10})$$

derived from the graph rewrite. Removing [X] from the mass-action equations using  $\dot{[X]} = 0$ , we derive that the rate constants satisfying Eq. (A9) are given by

$$\begin{aligned} k_{ab} &= \frac{k_a k_b}{\bar{k}_a + k_b} \\ \bar{k}_{ab} &= \frac{\bar{k}_a \bar{k}_b}{\bar{k}_a + k_b}. \end{aligned} \quad (\text{A11})$$

The associated equilibrium constant correctly satisfies the relation

$$\frac{k_{ab}}{\bar{k}_{ab}} = \frac{k_a k_b}{\bar{k}_a \bar{k}_b}. \quad (\text{A12})$$

#### b. Associativity of the elementary composition rule

The composition rule (A12) is associative, meaning that internal nodes may be removed from chains of reactions in any order, as shown in Fig. 20. All composition rules derived in the remainder of this appendix will be variants on the elementary rule (with additional buffered

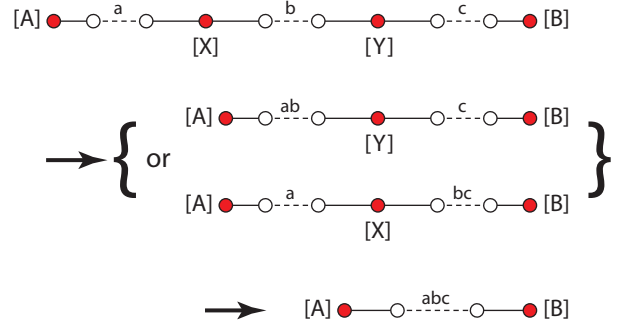


FIG. 20: Composition of three reactions  $a, b, c$  can proceed by elimination of either X or Y first.

concentration variables added), so we demonstrate associativity for the base case as the foundation for other cases.

From Eq. (A12) for  $(k_a, \bar{k}_a) \circ (k_b, \bar{k}_b)$ , followed by the equivalent expressions for  $(k_{ab}, \bar{k}_{ab}) \circ (k_c, \bar{k}_c)$ ,  $(k_a, \bar{k}_a) \circ (k_{bc}, \bar{k}_{bc})$ , and  $(k_b, \bar{k}_b) \circ (k_c, \bar{k}_c)$ , we derive the sequence of reductions

$$\begin{aligned} k_{abc} &= \frac{k_{ab} k_c}{\bar{k}_{ab} + k_c} \\ &= \frac{k_a k_b k_c}{\bar{k}_a \bar{k}_b + (\bar{k}_a + k_b) k_c} \\ &= \frac{k_a k_b k_c}{\bar{k}_a (\bar{k}_b + k_c) + k_b k_c} \\ &= \frac{k_a k_b k_c}{\bar{k}_a + k_{bc}}, \end{aligned} \quad (\text{A13})$$

and a similar equation follows for  $\bar{k}_{abc}$ . Thus we have

$$[(k_a, \bar{k}_a) \circ (k_b, \bar{k}_b)] \circ (k_c, \bar{k}_c) = (k_a, \bar{k}_a) \circ [(k_b, \bar{k}_b) \circ (k_c, \bar{k}_c)]. \quad (\text{A14})$$

#### c. Removal of internal nodes that require other inputs or outputs

Next we consider the elimination of an internal node [X] that is produced or consumed together with other products or reactants. Conservation  $\dot{[X]} = 0$  implies relations among the currents of these other species as well. All remaining graph reductions that we will perform for the TCA cycle are of this kind. In some cases both the secondary product and reactant are the solvent (water), as in the aconitase reactions (repeated in TCA, 3HB, 4HB, and bicycle pathways). In other cases they are reactants or inputs such as  $\text{CO}_2$  that we consider buffered in the environment.

The pair of mass action equations we wish to reduce

are<sup>60</sup>

$$\begin{aligned} [A] k_a - [X] [C] \bar{k}_a &= J_a \\ [X] [D] k_b - [B] \bar{k}_b &= J_b, \end{aligned} \quad (\text{A15})$$

and the desired reduced form is

$$[A] [D] k_{ab} - [C] [B] \bar{k}_{ab} = J_{ab}. \quad (\text{A16})$$

We first reduce Eq. (A15) to the base case of the previous section, by absorbing the concentrations not to be removed into a pair of effective rate constants

$$\begin{aligned} [A] k_a - [X] ([C] \bar{k}_a) &= J_a \\ [X] ([D] k_b) - [B] \bar{k}_b &= J_b. \end{aligned} \quad (\text{A17})$$

From these we derive a composition equation

$$[A] \hat{k}_{ab} - [B] \bar{\bar{k}}_{ab} = J_{ab}, \quad (\text{A18})$$

corresponding to the graph representation in Fig. 21. We may then define  $\hat{k}_{ab}$  and  $\bar{\bar{k}}_{ab}$  by the elementary composition rule (A10)

$$(k_a, [C] \bar{k}_a) \circ ([D] k_b, \bar{k}_b) = (\hat{k}_{ab}, \bar{\bar{k}}_{ab}), \quad (\text{A19})$$

giving the transformation<sup>61</sup>

$$\begin{aligned} \hat{k}_{ab} &= \frac{k_a [D] k_b}{[C] \bar{k}_a + [D] k_b} \\ \bar{\bar{k}}_{ab} &= \frac{[C] \bar{k}_a \bar{k}_b}{[C] \bar{k}_a + [D] k_b}. \end{aligned} \quad (\text{A20})$$

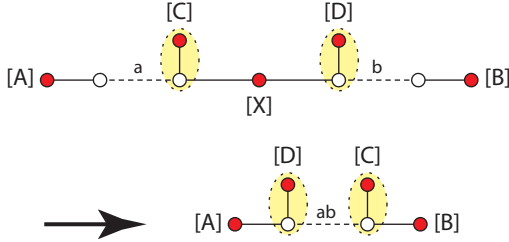


FIG. 21: Representation of a composite graph with internal connections other than those to X as an effective elementary graph. Highlights denote the absorption of other species into modifications of effective rate constants coupled to X at a and b. These are then used to define the elementary-form rate constants  $\hat{k}_{ab}$  and  $\bar{\bar{k}}_{ab}$  in the reduced graph.

Now removing the factors of [C] and [D] used to define the hatted rate constants,

$$\begin{aligned} \hat{k}_{ab} &= [D] k_{ab} \\ \bar{\bar{k}}_{ab} &= [C] \bar{k}_{ab}, \end{aligned} \quad (\text{A21})$$

we obtain a direct expression for the composition rule in Eq. (A18), of

$$\begin{aligned} k_{ab} &= \frac{k_a k_b}{[C] \bar{k}_a + [D] k_b} \\ \bar{k}_{ab} &= \frac{\bar{k}_a \bar{k}_b}{[C] \bar{k}_a + [D] k_b}, \end{aligned} \quad (\text{A22})$$

which is the interpretation of the graph reduction shown in Fig. 22.

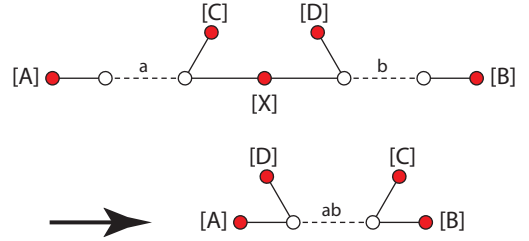


FIG. 22: The composite graph corresponding to the reduction from Eq. (A15) to Eq. (A16).

#### d. Associativity for composite graphs

Associativity for composite graphs follows from the associativity of the elementary composition rule (A14), via the grouping (A19). To show how this works, we demonstrate associativity for the minimal case shown in Fig. 23. The important features are that the graph “re-wiring” follows from composition of the rule demonstrated in Fig. 22, and the composition rule for rate constants permits consistent removal of the necessary factors of reagent concentrations.

The application of the elementary reduction to remove X, corresponding to the second line in Fig. 23, yields Eq’s. (A19,A20). An equivalent removal of Y first (the third line of Fig. 23) gives

$$(k_b, [E] \bar{k}_b) \circ ([F] k_c, \bar{k}_c) = (\hat{k}_{bc}, \bar{\bar{k}}_{bc}), \quad (\text{A23})$$

<sup>60</sup> In this and the following examples, we consider single additional species [C] and [D]. These may readily be generalized to a variety of cases in which the additional reagents are  $\prod_{k=1}^p [C_k]$  and  $\prod_{l=1}^q [D_l]$ .

<sup>61</sup> Note that if [C] and [D] are the same species these cancel in the numerator and denominator of Eq. (A20), and the same applies to common factors in products  $\prod_{k=1}^p [C_k]$  and  $\prod_{l=1}^q [D_l]$ . Therefore these factors may simply be removed before the graph reduction if desired, because they encoded redundant constraints with the conservation law already implied by  $[\dot{X}] = 0$ . The irrelevance of redundant species in the graph reduction for removal of [X] is radically different from the graphically similar-looking role of a network catalyst which is both an input and an output of the same reaction. Network catalysts are essential to the determination of reaction rates.



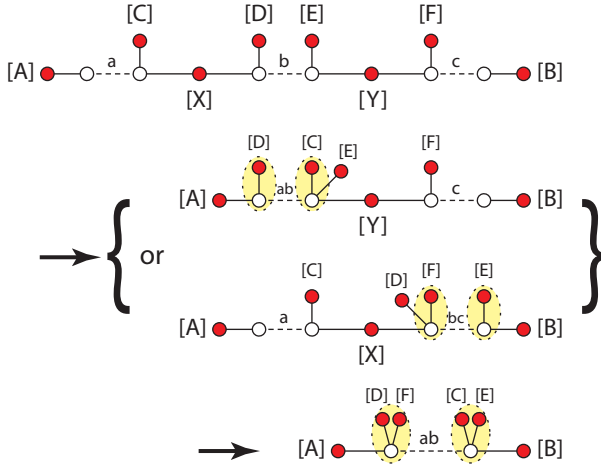


FIG. 23: A two-step reduction with other internal connections, which may be performed by removing either X or Y first.

with rule

$$\begin{aligned}\hat{k}_{bc} &= \frac{k_b [F] k_c}{[E] \bar{k}_b + [F] k_c} \\ \bar{k}_{bc} &= \frac{[E] \bar{k}_b \bar{k}_c}{[E] \bar{k}_b + [F] k_c}.\end{aligned}\quad (\text{A24})$$

The two equivalent rules for removing whichever internal node was not removed in the first reduction are

$$\begin{aligned}(\hat{k}_{ab}, [E] \bar{k}_{ab}) \circ ([F] k_c, \bar{k}_c) &= (\hat{k}_{abc}, \bar{k}_{abc}), \\ (k_a, [C] \bar{k}_a) \circ ([D] \hat{k}_{bc}, \bar{k}_{bc}) &= (\hat{k}_{abc}, \bar{k}_{abc}).\end{aligned}\quad (\text{A25})$$

Composing these rules for intermediate rate constants, we may check that

$$\begin{aligned}\hat{k}_{abc} &= \frac{\hat{k}_{ab} [F] k_c}{[E] \bar{k}_{ab} + [F] k_c} \\ &= \frac{(k_a [D] k_b) [F] k_c}{[C] \bar{k}_a [E] \bar{k}_b + ([C] \bar{k}_a + [D] k_b) [F] k_c} \\ &= \frac{k_a [D] (k_b [F] k_c)}{[C] \bar{k}_a ([E] \bar{k}_b + [F] k_c) + [D] k_b [F] k_c} \\ &= \frac{k_a [D] \hat{k}_{bc}}{[C] \bar{k}_a + [D] \hat{k}_{bc}},\end{aligned}\quad (\text{A26})$$

and a similar equation follows for  $\bar{k}_{abc}$ . Converting the hatted forms to the normal reaction form produces the rate equation

$$[A] [D] [F] k_{abc} - [C] [E] [B] \bar{k}_{abc} = J_{abc}.\quad (\text{A27})$$

We may directly obtain the rate constants  $k_{abc}$ ,  $\bar{k}_{abc}$  with the composition rule

$$\begin{aligned}(k_{abc}, \bar{k}_{abc}) &= (k_{ab}, \bar{k}_{ab}) \circ (k_c, \bar{k}_c) \\ &= (k_a, \bar{k}_a) \circ (k_{bc}, \bar{k}_{bc}),\end{aligned}\quad (\text{A28})$$

using the appropriate version of the graph-dependent evaluation rule (A22) in each step. The resulting composition (A28) is automatically associative, because it satisfies the conversion

$$\begin{aligned}\hat{k}_{abc} &= [D] [F] k_{abc} \\ \bar{k}_{abc} &= [C] [E] \bar{k}_{abc}\end{aligned}\quad (\text{A29})$$

with Eq. (A26), which is associative. As a final check, the equilibrium constants in the normal reaction form satisfy the necessary chain rule

$$\frac{k_{abc}}{\bar{k}_{abc}} = \frac{k_a k_b k_c}{\bar{k}_a \bar{k}_b \bar{k}_c}.\quad (\text{A30})$$

Intermediate (hatted) rate constants have been used here to show how associativity is inherited from the base case. The examples below work directly with the actual (unhatted) rate constants, which keep the network in its literal form at each reduction.

### 3. Application to the citric-acid cycle reactions

Using this graph representation and the associated graph reductions, we may express the qualitative kinetics associated with network autocatalysis in the rTCA cycle. We use a minimal model network in which only the cycle intermediates are represented explicitly, and only the CHO stoichiometry is retained.<sup>62</sup> External sources or sinks are used to buffer only four compounds in the network, which are CO<sub>2</sub>, H<sub>2</sub>, H<sub>2</sub>O, and a pool of reduced carbon which we take to be acetate (ACE, or CH<sub>3</sub>COOH) because it has the lowest free energy of formation of cycle intermediates under reducing conditions (following Ref. [266]) and is the natural drain compound [7].

The purpose of network reduction in such a model is to produce a graph in which each element corresponds to a specific control parameter for the interaction of conservation laws with non-equilibrium boundary conditions. CO<sub>2</sub>, H<sub>2</sub>, and H<sub>2</sub>O provide sources of carbon and reductant, and an output for reduced oxygen atoms. Because they comprise different ratios of three elements, any set of concentrations is consistent with a Gibbs equilibrium, and the chemical potentials corresponding to the elements are preserved by the conservation laws of arbitrary reactions. A fourth boundary condition for acetate cannot be linearly independent in equilibrium, and drives the steady-state reaction flux.

Such a model is limited in many ways. The replacement of explicit (and unknown) parasitic side reactions, from all cycle intermediates, by a single loss rate for acetate may fail to capture concentration-dependent losses,

<sup>62</sup> As noted above, phosphorylated intermediates and thioesters, including the energetically important substrate-level phosphorylation of citrate and succinate, are not represented.

in a way that cannot simply be absorbed into lumped rate constants. Moreover, the rate constants themselves depend on catalysts, and reasonable values for these in a prebiotic or early-cellular context are unknown. Therefore all critical properties of the model are expressed relative to these rate constants. The reduction remains meaningful, however, because the lumped-parameter rate constants are controlled by the three buffered environmental compounds  $\text{CO}_2$ ,  $\text{H}_2$ , and  $\text{H}_2\text{O}$ , leaving the network flux to be controlled by the disequilibrium concentration of acetate.

a. The graph reduction sequence

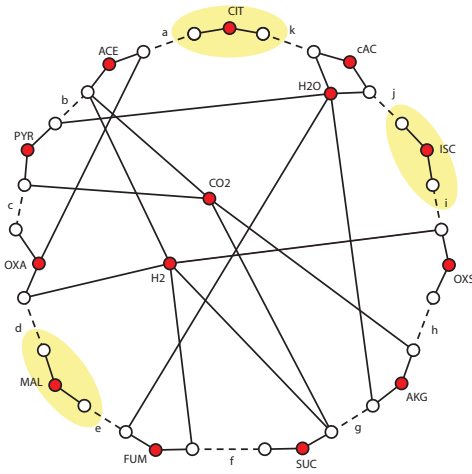


FIG. 24: The projection of the TCA cycle onto CHO compounds. Phosphates and thioesters are omitted, and the stoichiometry of all acids refers to the protonated forms, so that  $\text{H}_2$  stands for general two-electron reductants. Omission of explicit representations of substrate-level phosphorylation to form citryl-CoA and succinyl-CoA causes water elimination to accompany carboxylation of acetate and succinate in this graph, where in the actual cycle it would occur outside the graph, in the formation of pyrophosphates. Highlighted species are sole outputs and sole inputs of their associated reactions, and can be removed with the elementary composition rule (A11) of Sec. A 2 a. Legend: acetate (ACE), pyruvate (PYR), oxaloacetate (OXA), malate (MAL), fumarate (FUM), succinate (SUC),  $\alpha$ -ketoglutarate (AKG), oxalosuccinate (OXS), cis-aconitate (cAC), isocitrate (ISC), citrate (CIT).

The bipartite graph for the minimal rTCA network in CHO compounds is shown in Fig. 24. All networks in the text are generated by equivalent methods. Highlighted nodes are those that can be removed by the base reduction in Sec. A 2 a. Reactions are labeled with lower-case Roman letters, and relative to the elementary half-reaction rate constants, the lumped-parameter rate con-

stants are given by<sup>63</sup>

$$\begin{aligned} k_{de} &= \frac{k_d k_e}{\bar{k}_d + k_e} & \bar{k}_{de} &= \frac{\bar{k}_d \bar{k}_e}{\bar{k}_d + k_e} \\ k_{ij} &= \frac{k_i k_j}{\bar{k}_i + k_j} & \bar{k}_{ij} &= \frac{\bar{k}_i \bar{k}_j}{\bar{k}_i + k_j} \\ k_{ka} &= \frac{k_k k_a}{\bar{k}_k + k_a} & \bar{k}_{ka} &= \frac{\bar{k}_k \bar{k}_a}{\bar{k}_k + k_a}, \end{aligned} \quad (\text{A31})$$

with equivalent expressions for the  $\bar{k}$ s. These define the elementary reactions in the reduced graph of Fig. 25.

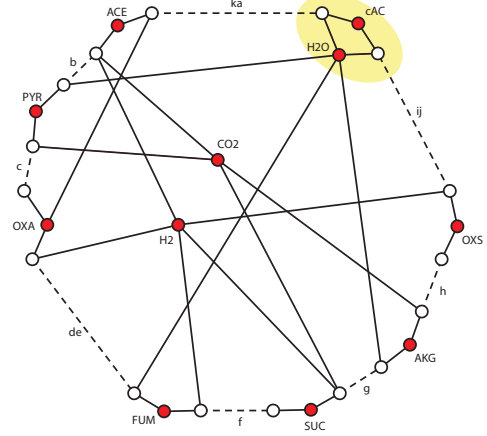


FIG. 25: Graph of Fig. 24 with its highlighted species removed. Cis-aconitate (cAC highlighted) has common factors of  $[\text{H}_2\text{O}]$ , and is the next internal node to be removed, by the rewrite rules of Sec. A 2 c, but with the simplifying feature that common factors cancel, so they resemble the base case.

One further reduction that follows the elementary rule in Fig. 25 is removal of cis-aconitate (cAC), which involves a common factor of the solvent  $[\text{H}_2\text{O}]$ . The resulting lumped-parameter rate constants are given by

$$k_{ijka} = \frac{k_{ij} k_{ka}}{\bar{k}_{ij} + k_{ka}} \quad \bar{k}_{ijka} = \frac{\bar{k}_{ij} \bar{k}_{ka}}{\bar{k}_{ij} + k_{ka}}. \quad (\text{A32})$$

These lead to the graph of Fig. 26.

All further graph reductions require the composition rules of Sec. A 2 c, and result in changes of the input or output stoichiometries of the unreduced nodes. All highlighted compounds in Fig. 26 may be removed, and the resulting lumped-parameter rate constants are given by

$$k_{bc} = \frac{k_b k_c}{[\text{H}_2\text{O}] \bar{k}_b + [\text{CO}_2] k_c}$$

<sup>63</sup> Here and below, we give formulae only for the forward half-reaction rate constants  $k$ . Formulae for the backward half-reaction rate constants  $\bar{k}$  have corresponding forms as shown in the preceding sections.

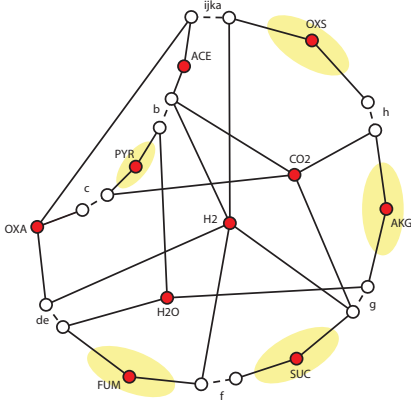


FIG. 26: Graph of Fig. 25 with cAC and its parallel links to water removed. For all remaining species except acetate (ACE), neither sources nor sinks are assumed, and these may be removed with non-trivial instances of the composition rule of Sec. A 2 c. Each of these removals changes the degree of the remaining reactions, and thus changes the topology of the graph.

$$\begin{aligned}
 k_{def} &= \frac{k_{de}k_f}{[\text{H}_2\text{O}]k_{de} + [\text{H}_2]k_f} \\
 k_{defg} &= \frac{k_{def}k_g}{[\text{H}_2\text{O}]\bar{k}_{def} + [\text{H}_2][\text{CO}_2]k_g} \\
 k_{defgh} &= \frac{k_{defg}k_h}{[\text{H}_2\text{O}]^2\bar{k}_{defg} + [\text{CO}_2]k_h} \\
 k_{defghijka} &= \frac{k_{defgh}k_{ijka}}{[\text{H}_2\text{O}]^2\bar{k}_{defgh} + [\text{H}_2]k_{ijka}}. \quad (\text{A33})
 \end{aligned}$$

These define the maximal reduction of the original rTCA graph, to the graph shown in Fig. 27.

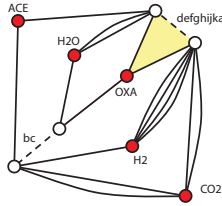


FIG. 27: Graph of Fig. 26 with all internal nodes from linear chains removed.  $[\text{H}_2\text{O}]$ ,  $[\text{H}_2]$ ,  $[\text{CO}_2]$ , and  $[\text{ACE}]$  are the four molecular concentrations to which boundary sources are coupled.  $[\text{OXA}]$  is retained as the last representation of the network catalysis of the loop, indicated by highlighting of the reaction in which OXA is input and output with equal stoichiometry. In steady state, OXA is in equilibrium with ACE, because it is not coupled to external currents.

The lumped-parameter rate equations for Fig. 27, parametrized by lumped-parameter rate constants, are

$$\begin{aligned}
 J_{bc} &= [\text{ACE}][\text{H}_2][\text{CO}_2]^2 k_{bc} \\
 &\quad - [\text{OXA}][\text{H}_2\text{O}]\bar{k}_{bc} \\
 J_{defghijka} &= [\text{OXA}][\text{H}_2]^4[\text{CO}_2]^2 k_{defghijka} \\
 &\quad - [\text{OXA}][\text{ACE}][\text{H}_2\text{O}]^2 \bar{k}_{defghijka} \quad (\text{A34})
 \end{aligned}$$

In steady state  $J_{bc} = 0$  and  $[\text{OXA}]$  may be replaced with the equilibrium function

$$[\text{OXA}] = \frac{k_{bc}}{\bar{k}_{bc}} \frac{[\text{H}_2][\text{CO}_2]^2}{[\text{H}_2\text{O}]} [\text{ACE}]. \quad (\text{A35})$$

#### b. Network reaction fluxes and their control parameters

For the remainder of the appendix we replace the subscript  $defghijka$  with designation  $r_{\text{TCA}}$  in currents  $J$ , half-reaction rate constants  $k$ ,  $\bar{k}$ , and equilibrium constants  $K$ . Dimensionally, the rate constants require the concentration of OXA in the mass-action law, and so presume that the anaplerotic segment  $bc$  has been handled.

Plugging Eq. (A35) into the second rate equation of Eq. (A34), and supposing  $[\text{OXA}]$  is in equilibrium with  $[\text{ACE}]$  at a (non-equilibrium) steady state for the network as a whole, we obtain the only independent mass-action rate equation for the reduced network. This is the current producing acetate:

$$J_{r_{\text{TCA}}} = \bar{k}_{r_{\text{TCA}}} \frac{k_{bc}}{\bar{k}_{bc}} [\text{H}_2][\text{CO}_2]^2 [\text{H}_2\text{O}][\text{ACE}] \left( \frac{k_{r_{\text{TCA}}}}{\bar{k}_{r_{\text{TCA}}}} \frac{[\text{H}_2]^4[\text{CO}_2]^2}{[\text{H}_2\text{O}]^2} - [\text{ACE}] \right). \quad (\text{A36})$$

The first term in parenthesis in Eq. (A36) is the concentration at which acetate would be in equilibrium with the

inorganic inputs, which we denote<sup>64</sup>

$$[\text{ACE}]_G \equiv \frac{k_{r_{\text{TCA}}}}{\bar{k}_{r_{\text{TCA}}}} \frac{[\text{H}_2]^4[\text{CO}_2]^2}{[\text{H}_2\text{O}]^2}. \quad (\text{A37})$$

<sup>64</sup> Although the lumped-parameter rate constant in this relation appears complex, the consistency conditions with single-reaction

Therefore the network response is proportional to the offset of  $[ACE]$  from its equilibrium value, with a rate constant that depends on the particular contributions of chemical potential from  $[CO_2]$  and reductant.

#### 4. Interaction of Wood-Ljungdahl with rTCA

We may envision an early Wood-Ljungdahl “feeder” pathway to acetyl-CoA as a reaction with the same stoichiometry as rTCA for the creation of acetate, but fixed half-reaction rate constants that do not depend on the internal concentrations in the network. This may be a pre-pterin mineral pathway [56], in which rate constants are determined by the abiotic environment, or an early pathway using pterin-like cofactors, if the concentrations of these are somehow buffered from the instantaneous flows through the reductive pathway. Labeling this “linear” effective reaction WL, the rate equation becomes

$$J_{WL} = \bar{k}_{WL} [H_2O]^2 \left( \frac{k_{WL} [H_2]^4 [CO_2]^2}{\bar{k}_{WL} [H_2O]^2} - [ACE] \right). \quad (A38)$$

equilibrium constants ensure that  $k_{rTCA}/\bar{k}_{rTCA}$  is independent of synthetic pathway and equal to the exponential of the Gibbs

Note that  $k_{WL}/\bar{k}_{WL} = k_{rTCA}/\bar{k}_{rTCA}$  because both are expressions for the equilibrium constant which depends only on the free energy of reaction.

To understand the performance of a joint network in the presence of losses, as the simplest case introduce a reaction labeled Env standing for dilution of acetate to an environment at zero concentration. The dilution current becomes

$$J_{Env} = k_{Env} [ACE]. \quad (A39)$$

At a non-equilibrium steady state the total losses must equal the total supply currents, so that

$$J_{Env} = J_{rTCA} + J_{WL}. \quad (A40)$$

The un-reduced equation for steady-state currents can be written  
free energy of formation.

$$\begin{aligned} J_{rTCA} + J_{WL} &= [H_2O]^2 \left\{ \sqrt{k_{rTCA} \bar{k}_{rTCA}} \frac{K_{bc}}{K_{rTCA}} \frac{[CO_2]}{[H_2]} [ACE]^{1/2} [ACE] + \bar{k}_{WL} \right\} ([ACE]_G - [ACE]) \\ &= J_D = k_D [ACE] \end{aligned} \quad (A41)$$

The graph corresponding to this model for rate laws is shown in Fig. 28.

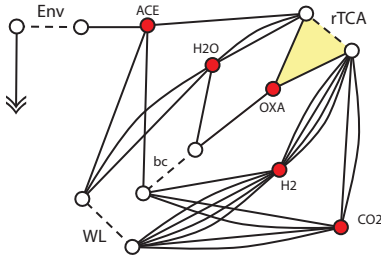


FIG. 28: Hypergraph model for parallel reactions through the rTCA and WL pathways, coupled to a linear drain reaction representing dilution of acetate by the environment.

The variable that characterizes the “impedance” of a chemical reaction network, and displays thresholds for autocatalysis when these exist, is the ratio of the output acetate concentration to the value that would exist in a

Gibbs equilibrium with the inputs:

$$x \equiv \frac{[ACE]}{[ACE]_G}. \quad (A42)$$

For a network with no reaction barriers (either in rate constants or due to limitations of network catalysts, the output  $x \rightarrow 1$ .

The two control parameters that govern the relative contributions of the rTCA loop and the direct WL feeder are

$$\begin{aligned} z_{rTCA} &= \frac{\sqrt{k_{rTCA} \bar{k}_{rTCA}}}{k_{Env}} \frac{K_{bc}}{K_{rTCA}} \frac{[CO_2] [H_2O]^2}{[H_2]} [ACE]_G^{3/2} \\ z_{WL} &= \frac{\bar{k}_{WL} [H_2O]^2}{k_{Env}}. \end{aligned} \quad (A43)$$

Each control parameter is a ratio of lumped half-reaction rates that feed  $[ACE]$  to the environment dilution constant  $k_{Env}$  through which it drains.

In terms of  $z_{WL}$  and  $z_{rTCA}$ , the normalized concentration  $x$  – which is proportional by  $k_{Env}$  to the total current

through the system – satisfies

$$x = \frac{1}{2} \left( 1 - \frac{1 + z_{\text{WL}}}{z_{\text{TCA}}} \right) + \sqrt{\frac{z_{\text{WL}}}{z_{\text{TCA}}} + \frac{1}{4} \left( 1 - \frac{1 + z_{\text{WL}}}{z_{\text{TCA}}} \right)^2}. \quad (\text{A44})$$

The solution to Eq. (A44) is shown versus base-10 logarithms of  $z_{\text{TCA}}$  and  $z_{\text{WL}}$  in Fig. 12 in the main text.

The critical (unsupported) response of the rTCA loop occurs at  $z_{\text{WL}} \rightarrow 0$  and  $z_{\text{TCA}} = 1$ . It is identified with the discontinuity in the derivative  $\partial x / \partial z_{\text{TCA}}$  at  $z_{\text{TCA}} = 1$  and the exactly zero value of  $x$  for  $z_{\text{TCA}} < 1$ . As  $z_{\text{WL}}$  increases from zero, the transition becomes smooth, and a nonzero concentration  $x$  is maintained against dilution at *all* values of  $z_{\text{TCA}}$ .

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- [1] E. Schrödinger. *What is Life?: the physical aspect of the living cell*. Cambridge U. Press, New York, 1992.
  - [2] Eric Smith. Thermodynamics of natural selection I: Energy and entropy flows through non-equilibrium ensembles. *J. Theor. Biol.*, <http://dx.doi.org/10.1016/j.jtbi.2008.02.010>, 2008.
  - [3] Eric Smith. Thermodynamics of natural selection II: Chemical Carnot cycles. *J. Theor. Biol.*, <http://dx.doi.org/10.1016/j.jtbi.2008.02.008>, 2008.
  - [4] Paul G. Falkowski, Tom Fenchel, and Edward F. DeLong. The microbial engines that drive earth’s biogeochemical cycles. *Science*, 320:1034–1039, 2008.
  - [5] Joseph W. Lengeler, Gerhart Drews, and Hans G. Schlegel. *Biology of the Prokaryotes*. Blackwell Science, New York, 1999.
  - [6] Harold J. Morowitz. *Beginnings of Cellular Life*. Yale U. Press, New Haven, CT., 1992.
  - [7] Eric Smith and Harold J. Morowitz. Universality in intermediary metabolism. *Proc. Nat. Acad. Sci. USA*, 101:13168–13173, 2004. SFI preprint # 04-07-024.
  - [8] Kalervo Rankama and Thure Georg Sahama. *Geochemistry*. U. Chicago Press, Chicago, Ill., 1950.
  - [9] Arren Bar-Even, Elad Noor, Yonatan Savir, Wolfram Liebermeister, Dan Davidi, Dan S. Tawfik, and Ron Milo. The moderately efficient enzyme: Evolutionary and physicochemical trends shaping enzyme parameters. *Biochem.*, 50:4402–4410, 2011.
  - [10] Herbert A. Simon. The architecture of complexity. *Proc. Am. Phil. Soc.*, 106:467–482, 1962.
  - [11] Herbert A. Simon. The organization of complex systems. In Howard H. Pattee, editor, *Hierarchy theory: The challenge of complex systems*, pages 3–27, New York, 1973. George Braziller.
  - [12] L. W. Ance and W. Fontana. Plasticity, evolvability and modularity in rna. *J. Exp. Zool. (Mol. Dev. Evol.)*, 288:242–283, 2000.
  - [13] W. Fontana. Modeling ‘evo-devo’ with rna. *Bioessays*, 24:1164–1177, 2002.
  - [14] Andreas Wagner. Robustness and evolvability: a paradox resolved. *Proceedings of the Royal Society B: Biological Sciences*, 275(1630):91–100, 2008.
  - [15] Gunter P. Wagner and Lee Altenberg. Perspective: Complex adaptations and the evolution of evolvability. *Evolution*, 50(3):967–976, 1996.
  - [16] Marc Kirschner and John Gerhart. Evolvability. *Proceedings of the National Academy of Sciences*, 95(15):8420–8427, 1998.
  - [17] John Gerhart and Marc Kirschner. *Cells, embryos, and evolution*. Wiley, New York, 1997.
  - [18] John Gerhart and Marc Kirschner. The theory of facilitated variation. *Proc. Nat. Acad. Sci. USA*, 104:8582–8589, 2007.
  - [19] E. Ravasz, A. L. Somera, D. A. Mongru, Z. N. Oltvai, and A.-L. Barabási. Hierarchical organization of modularity in metabolic networks. *Science*, 297(5586):1551–1555, 2002.
  - [20] Roger Guimera and Luis A. Nunes Amaral. Functional cartography of complex metabolic networks. *Nature*, 433(7028):895–900, 2005.
  - [21] M. E. J. Newman. Modularity and community structure in networks. *Proceedings of the National Academy of Sciences*, 103(23):8577–8582, 2006.
  - [22] J. J. Elser, R. W. Sterner, E. Gorokhova, W. F. Fagan, T. A. Markow, J. B. Cotner, J. F. Harrison, S. E. Hobbie, G. M. Odell, and L. W. Weider. Biological stoichiometry from genes to ecosystems. *Ecology Letters*, 3:540–550, 2000.
  - [23] J. Craig Venter, Karin Remington, John F. Heidelberg, Aaron L. Halpern, Doug Rusch, Jonathan A. Eisen, Dongying Wu, Ian Paulsen, Karen E. Nelson, William Nelson, Derrick E. Fouts, Samuel Levy, Anthony H. Knap, Michael W. Lomas, Ken Nealson, Owen White, Jeremy Peterson, Jeff Hoffman, Rachel Parsons, Holly Baden-Tillson, Cynthia Pfannkoch, Yu-Hui Rogers, and Hamilton O. Smith. Environmental genome shotgun sequencing of the sargasso sea. *Science*, 304:66–74, 2004.
  - [24] Elhanan Borenstein, Martin Kuplic, Marcus W. Feldman, and Eytan Ruppin. Large-scale reconstruction and phylogenetic analysis of metabolic environments. *Proc. Nat. Acad. Sci. USA*, 105:14482–14487, 2008.
  - [25] Jan P. Amend and Everett L. Shock. Energetics of overall metabolic reactions of thermophilic and hyperthermophilic archaea and bacteria. *FEMS Microbiology Reviews*, 25:175–243, 2001.
  - [26] Anna-Louise Reysenbach and Everett Shock. Merging genomes with geochemistry in hydrothermal ecosystems. *Science*, 296:1077–1082, 2002.
  - [27] William Martin, John Baross, Deborah Kelley, and Michael J. Russell. Hydrothermal vents and the origin of life. *Nature Rev. Microbiol.*, 6:805–814, 2008.
  - [28] Everett L. Shock. Minerals as energy sources for microorganisms. *Econ. Geol.*, 104:1235–1248, 2009.
  - [29] Douglas H. Erwin, Marc Laflamme, Sarah M. Tweedt, Erik A. Sperling, Davide Pisani, and Kevin J. Peterson. The Cambrian conundrum: early divergence and later ecological success in the early history of animals. *Science*, 334:1091–1097, 2011.
  - [30] Douglas H. Erwin and Sarah Tweedt. Ecological drivers of the Ediacaran-Cambrian diversification of metazoa. *Evol. Ecol.*, 26:417–433, 2012.
  - [31] Douglas H. Erwin and James W. Valentine. *The Cambrian Radiation*. in press, 2012.

- [32] Douglas H. Erwin. Macroevolution: dynamics of diversity. *Current Biology*, 21:R1000–R1001, 2012.
- [33] Harold J. Morowitz and Eric Smith. Energy flow and the organization of life. *Complexity*, 13:51–59, 2007. SFI preprint # 06-08-029.
- [34] Jo/ao F. Matias Rodrigues and Andreas Wagner. Evolutionary plasticity and innovations in complex metabolic reaction networks. *PLoS Computational Biology*, 5:e1000613:1–11, 2009.
- [35] Leo W. Buss. *The evolution of individuality*. Princeton U. Press, Princeton, N. J., 2007.
- [36] R. A. Fisher. *The genetical theory of natural selection*. Oxford U. Press, London, 2000.
- [37] Warren J. Ewens. *Mathematical Population Genetics*. Springer, Heidelberg, second edition, 2004.
- [38] F. John Odling-Smee, Kevin N. Laland, and Marcus W. Feldman. *Niche construction: the neglected process in evolution*. Princeton U. Press, Princeton, N. J., 2003.
- [39] Vijayarathy Srinivasan and Harold J. Morowitz. The canonical network of autotrophic intermediary metabolism: Minimal metabolome of a reductive chemoautotroph. *Biol. Bulletin*, 216:126–130., 2009.
- [40] Vijayarathy Srinivasan and Harold J. Morowitz. Analysis of the intermediary metabolism of a reductive chemoautotroph. *Biol. Bulletin*, 217:222–232, 2009.
- [41] Rogier Braakman and Eric Smith. The emergence and early evolution of biological carbon fixation. *PLoS Comp. Biol.*, 8:e1002455, 2012.
- [42] Marie Csete and John Doyle. Bow ties, metabolism and disease. *Trends. Biotechnol.*, 22:446–450, 2004.
- [43] Jing Zhao, Lin Tao, Hong Yu, JianHua Luo, ZhiWei Cao, and YiXue Li. Bow-tie topological features of metabolic networks and the functional significance. *Chinese Sci. Bull.*, 52:1036–1045, 2007.
- [44] William J. Riehl, Paul L. Krapivsky, Sidney Redner, and Daniel Segrè. Signatures of arithmetic simplicity in metabolic network architecture. *PLoS Computational Biology*, 6:e1000725, 2010.
- [45] Vijayarathy Srinivasan, Harold J. Morowitz, and Harold Huber. What is an autotroph? *Arch. Microbiol.*, 194:135–140, 2012.
- [46] Robert W. Sterner and James J. Elser. *Ecological Stoichiometry: the biology of elements from molecules to the biosphere*. Princeton U. Press, Princeton, NJ, 2002.
- [47] Eric Smith and Harold J. Morowitz. The autotrophic origins paradigm and small-molecule organocatalysis. *Orig. Life Evol. Biosphere*, 40:397–402, 2010.
- [48] Rafael F. Say and Georg Fuchs. Fructose 1,6-bisphosphate aldolase/phosphatase may be an ancestral gluconeogenic enzyme. *Nature*, 464:1077–1081, 2010.
- [49] Alexander I. Oparin. The origin of life. In Bernal [267], pages 199–234.
- [50] J. B. S. Haldane. The origin of life. In Bernal [267], pages 242–249.
- [51] Carl R. Woese. The universal ancestor. *Proc. Nat. Acad. Sci. USA*, 95:6854–6859, 1998.
- [52] C. R. Woese. Interpreting the universal phylogenetic tree. *Proc. Nat. Acad. Sci. USA*, 97:8392–8396, 2000.
- [53] C. R. Woese. On the evolution of cells. *Proc. Nat. Acad. Sci. USA*, 99:8742–8747, 2002.
- [54] Nigel Goldenfeld and Carl Woese. Life is physics: evolution as a collective phenomenon far from equilibrium. *Ann. Rev. Cond. Matt. Phys.*, 2010.
- [55] Steven A. Benner, Andrew D. Ellington, and Andreas Tauer. Modern metabolism as a palimpsest of the rna world. *Proc. Nat. Acad. Sci. USA*, 86:7054–7058, 2006.
- [56] Claudia Huber and Wächtershäuser, Günter. Activated acetic acid by carbon fixation on (fe,ni)s under primordial conditions. *Science*, 276:245–247, 2000.
- [57] Manfred Eigen and Peter Schuster. The hypercycle, part a: The emergence of the hypercycle. *Naturwissenschaften*, 64:541–565, 1977.
- [58] Manfred Eigen and Peter Schuster. The hypercycle, part c: The realistic hypercycle. *Naturwissenschaften*, 65:341–369, 1978.
- [59] Stuart Kauffman. *The Origins of Order: Self-Organization and Selection in Evolution*. Oxford U. Press, London, 1993.
- [60] Wim Hordijk, Stuart A. Kauffman, and Mike Steel. Required levels of catalysis for emergence of autocatalytic sets in models of chemical reaction systems. *Int. J. Mol. Sci.*, 12:3085–3101, 2012.
- [61] Jakob L. Andersen, Christoph Flamm, Daniel Merkle, and Peter F. Stadler. Maximizing output and recognizing autocatalysis in chemical reaction networks is NP-complete. *J. Sys. Chem.*, 3:1, 2012.
- [62] M. F. Utter and H. G. Wood. Mechanisms of fixation of carbon dioxide by heterotrophs and autotrophs. *Adv. Enzymol. Relat. Areas Mol. Biol.*, 12:41–151, 1951.
- [63] L. Ljungdahl, E. Irion, and H. G. Wood. Total synthesis of acetate from co<sub>2</sub>. i. co-methylcobyrilic acid and co-(methyl)-5-methoxybenzimidazolylcobamide as intermediates with *Clostridium thermoaceticum*. *Biochemistry*, 4:2771–2780, 1965.
- [64] L. Ljungdahl and H. G. Wood. Incorporation of c<sup>14</sup> from carbon dioxide into sugar phosphates, carboxylic acids, and amino acids by *Clostridium thermoaceticum*. *J. Bacteriol.*, 89:1055–1064, 1965.
- [65] B. Edward H. Maden. Tetrahydrofolate and tetrahydromethanopterin compared: functionally distinct carriers in c<sub>1</sub> metabolism. *Biochem. J.*, 350:609–629, 2000.
- [66] Ivan A. Berg, Daniel Kockelkorn, W. Hugo Ramos-Vera, Rafael F. Say, Jan Zarzycki, Michael Hügler, Birgit E. Alber, and Georg Fuchs. Autotrophic carbon fixation in archaea. *Nature Rev. Microbiol.*, 8:447–460, 2010.
- [67] Juli Pereto. Out of fuzzy chemistry: from prebiotic chemistry to metabolic networks. *Chem. Soc. Rev.*, pages –, 2012.
- [68] Michael J. Russell and William Martin. The rocky roots of the acetyl-coa pathway. *Trends Biochem. Sci.*, 29:358–363, 2004.
- [69] William Martin and Michael J. Russell. On the origin of biochemistry at an alkaline hydrothermal vent. *Phil. Trans. Roy. Soc. B*, 362:1887–1926, 2007.
- [70] B. B. Buchanan and D. I. Arnold. A reverse krebs cycle in photosynthesis: Consensus at last. *Photosynth. Res.*, 24:47–53, 1990.
- [71] George D. Cody, Nabil Z. Boctor, Timothy R. Filley, Robert M. Hazen, James H. Scott, Anurag Sharma, and Hatten S. Jr. Yoder. Primordial carbonylated iron-sulfur compounds and the synthesis of pyruvate. *Science*, 289:1337–1340, 2000.
- [72] Michael Hügler and S. M. Seivert. Beyond the calvin cycle: Autotrophic carbon fixation in the ocean. *Ann. Rev. Marine Sci.*, 3:261–289, 2011.
- [73] Georg Fuchs. Alternative pathways of carbon dioxide fixation: Insights into the early evolution of life? *Ann. Rev. Microbiol.*, 65(1):631–658, 2011.



- [74] Arren Bar-Even, Avi Flamholz, Elad Noor, and Ron Milo. Thermodynamic constraints shape the structure of carbon fixation pathways. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1817(9):1646 – 1659, 2012.
- [75] Holger Dobbek, Vitali Svetlitchnyi, Lothar Gremer, Robert Huber, and Ortwin Meyer. Crystal structure of a carbon monoxide dehydrogenase reveals a [Ni-4Fe-5S] cluster. *Science*, 293:1281–1285, 2001.
- [76] Claudine Darnault, Anne Volbeda, Eun Jin Kim, Pierre Legrand, Xavier Vernede, Paul A. Lindahl, and Juan C. Fontecilla-Camps. Ni-zn-[fe4-s4] and ni-ni-[fe4-s4] clusters in closed and open [alpha] subunits of acetyl-coa synthase/carbon monoxide dehydrogenase. *Nat Struct Mol Biol*, 10(4):271–279, 2003.
- [77] Javier Seravalli, Yuming Xiao, Weiwei Gu, Stephen P. Cramer, William E. Antholine, Vladimir Krymov, Gary J. Gerfen, and Stephen W. Ragsdale. Evidence that nini acetyl-coa synthase is active and that the cuni enzyme is not. *Biochemistry*, 43(13):3944–3955, 2004.
- [78] Vitali Svetlitchnyi, Holger Dobbek, Wolfram Meyer-Klaucke, Thomas Meins, Brbel Thiele, Piero Rmer, Robert Huber, and Ortwin Meyer. A functional nini-[4fe-4s] cluster in the monomeric acetyl-coa synthase from carboxydotherrmus hydrogenoformans. *Proceedings of the National Academy of Sciences of the United States of America*, 101(2):446–451, 2004.
- [79] Ruma Banerjee and Stephen W. Ragsdale. The many faces of vitamin b12: catalysis by cobalamin-dependent enzymes. *Annu. Rev. Biochem.*, 72:209–247, 2003.
- [80] Gunes Bender, Elizabeth Pierce, Jeffrey A. Hill, Joseph E. Darty, and Stephen W. Ragsdale. Metal centers in the anaerobic microbial metabolism of co and co2. *Metallomics*, 3:797–815, 2011.
- [81] S. W. Ragsdale, J. E. Clark, L. G. Ljungdahl, L. L. Lundie, and H. L. Drake. Properties of purified carbon monoxide dehydrogenase from clostridium thermoaceticum, a nickel, iron-sulfur protein. *Journal of Biological Chemistry*, 258(4):2364–2369, 1983.
- [82] Jan Zarzycki, Volker Brecht, Michael Müller, and Georg Fuchs. Identifying the missing steps of the autotrophic 3-hydroxypropionate CO<sub>2</sub> fixation cycle in *Chloroflexus aurantiacus*. *Proc. Nat. Acad. Sci. USA*, 106:21317–21322, 2009.
- [83] J. Dongun Kim, Augustina Rodriguez-Granillo, David A. Case, Vikas Nanda, and Paul G. Falkowski. Energetic selection of topology in ferredoxins. *PLoS comp. biol.*, 8:e1002463, 2012.
- [84] E. Chabrière, M. H. Charon, A. Volbeda, L. Pieulle, E. C. Hatchikian, and J. C. Fontecilla-Camps. Crystal structures of the key anaerobic enzyme pyruvate:ferredoxin oxidoreductase, free and in complex with pyruvate. *Nat. Struct. Biol.*, 6:182–190, 1999.
- [85] Jonathan Lombard and David Moreira. Early evolution of the biotin-dependent carboxylase family. *BMC evol. biol.*, 11:232:1–22, 2011.
- [86] Miho Aoshima, Masaharu Ishii, and Yasuo Igarashi. A novel biotin protein required for reductive carboxylation of 2-oxoglutarate by isocitrate dehydrogenase in *Hydrogenobacter thermophilus* TK-6. *Mol. Microbiol.*, 51:791–798, 51.
- [87] M. C. W. Evans, B. B. Buchanan, and D. I. Arnon. A new ferredoxin dependent carbon reduction cycle in photosynthetic bacterium. *Proc. Nat. Acad. Sci. USA*, 55:928–934, 1966.
- [88] Günter Wächtershäuser. Evolution of the first metabolic cycles. *Proc. Nat. Acad. Sci. USA*, 87:200–204, 1990.
- [89] Miho Aoshima, Masaharu Ishii, and Yasuo Igarashi. A novel enzyme, citryl-coa synthetase, catalysing the first step of the citrate cleavage reaction in hydrogenobacter thermophilus tk-6. *Molecular Microbiology*, 52:751–761, 2004.
- [90] Miho Aoshima, Masaharu Ishii, and Yasuo Igarashi. A novel enzyme, citryl-coa lyase, catalysing the second step of the citrate cleavage reaction in hydrogenobacter thermophilus tk-6. *Molecular Microbiology*, 52:763–770, 2004.
- [91] Shinya Watanabe, Michael Zimmermann, Michael B. Goodwin, Uwe Sauer, Clifton E. Barry 3rd, and Helena I. Boshoff. Fumarate reductase activity maintains an energized membrane in anaerobic *Mycobacterium tuberculosis*. *PLoS Pathogens*, 7:e1002287, 2011.
- [92] Jing Tian, Ruslana Bryk, Manabu Itoh, Makoto Suematsu, and Carl Nathan. Variant tricarboxylic acid cycle in *Mycobacterium tuberculosis*: identification of  $\alpha$ -ketoglutarate decarboxylase. *Proc. Nat. Acad. Sci. USA*, 102:10670–10675, 2005.
- [93] Baughn, Anthony D. and Garforth, Scott J. and Vilchère, Catherine and Jacobs Jr., William R. An anaerobic-type  $\alpha$ -ketoglutarate ferredoxin oxidoreductase completes the oxidative tricarboxylic acid cycle of *Mycobacterium tuberculosis*. *PLoS Pathogens*, 5:e1000662:1–10, 2009.
- [94] Shuyi Zhang and Donald A. Bryant. The tricarboxylic acid cycle in cyanobacteria. *Science*, 334:1551–1553, 2011.
- [95] Harald Huber, Martin Gallenberger, Ulrike Jahn, Eva Eylert, Ivan A. Berg, Daniel Kockelkorn, Wolfgang Eisenreich, and Georg Fuchs. A dicarboxylate/4-hydroxybutyrate autotrophic carbon assimilation cycle in the hyperthermophilic archaeum *Ignicoccus hospitalis*. *Proc. Nat. Acad. Sci. USA*, 105:7851–7856, 2008.
- [96] Ute Müh, Irfan Cinkaya, Simon P. J. Albracht, and Wolfgang Buckel. 4-hydroxybutyryl-coa dehydratase from clostridium aminobutyricum: Characterization of fad and ironsulfur clusters involved in an overall non-redox reaction. *Biochemistry*, 35(36):11710–11718, 1996.
- [97] Berta M. Martins, Holger Dobbek, Irfan Cinkaya, Wolfgang Buckel, and Albrecht Messerschmidt. Crystal structure of 4-hydroxybutyryl-coa dehydratase: Radical catalysis involving a [4fe-4s] cluster and flavin. *Proceedings of the National Academy of Sciences of the United States of America*, 101(44):15645–15649, 2004.
- [98] Birgit E. Alber, Johannes W. Kung, and Georg Fuchs. 3-hydroxypropionyl-coenzyme a synthetase from metallosphaera sedula, an enzyme involved in autotrophic co2 fixation. *Journal of Bacteriology*, 190(4):1383–1389, February 15, 2008.
- [99] Robin Teufel, Johannes W. Jung, Daniel Kockelkorn, Birgit E. Alber, and Georg Fuchs. 3-Hydroxypropionyl-Coenzyme A Dehydratase and Acrolyl-Coenzyme A Reductase, Enzymes of the Autotrophic 3-Hydroxypropionate/4-Hydroxybutyrate Cycle in the *Sulfolobales*. *J. Bacteriology*, 191:4572–4581, 2009.
- [100] J. A. Bassham, A. A. Benson, L. D. Kay, A. Z. Harris, A. T. Wilson, and M. Calvin. The path of carbon in photosynthesis. xxi. the cyclic regeneration of carbon dioxide acceptor. *J. Am. Chem. Soc.*, 76:1760–1770,

- 1954.
- [101] F. A. Tabita. Research on carbon dioxide fixation in photosynthetic microorganisms (1971 – present). *Photosynth. Res.*, 80:315–332, 2004.
  - [102] Marion Eisenhut, Shira Kahlon, Dirk Hasse, Ralph Ewald, Judy Lieman-Hurwitz, Teruo Ogawa, Wolfgang Ruth, Hermann Bauwe, Aaron Kaplan, and Martin Hagemann. The plant-like c2 glycolate cycle and the bacterial-like glycerate pathway cooperate in phosphoglycolate metabolism in cyanobacteria. *Plant Physiology*, 142:333–342, 2006.
  - [103] Marion Eisenhut, Wolfgang Ruth, Maya Haimovich, Hermann Bauwe, Aaron Kaplan, and Martin Hagemann. The photorespiratory glycolate metabolism is essential for cyanobacteria and might have been conveyed endosymbiotically to plants. *Proc. Nat. Acad. Sci. USA*, 105(44):17199–17204, 2008.
  - [104] Christine H. Foyer, Arnold J. Bloom, Guillaume Queval, and Graham Noctor. Photorespiratory metabolism: Genes, mutants, energetics, and redox signaling. *Annu. Rev. Plant Biol.*, 60:455–484, 2009.
  - [105] Yongqing Liu, Jizhong Zhou, Marina V. Omelchenko, Alex S. Beliaev, Amudhan Venkateswaran, Julia Stair, Liyou Wu, Dorothea K. Thompson, Dong Xu, Igor B. Rogozin, Elena K. Gaidamakova, Min Zhai, Kira S. Makarova, Eugene V. Koonin, and Michael J. Daly. Transcriptome dynamics of deinococcus radiodurans recovering from ionizing radiation. *Proceedings of the National Academy of Sciences*, 100(7):4191–4196, 2003.
  - [106] D. von Wettstein, S. Gough, and C. G. Kannangara. Chlorophyll biosynthesis. *The Plant Cell Online*, 7(7):1039–1057, 1995.
  - [107] Rogier Braakman and Eric Smith. An evolutionary context for the reconstructed metabolism of the hyperthermophilic chemoautotroph *Aquifex aeolicus*. 2012.
  - [108] Eric H. Davidson and Douglas H. Erwin. Gene regulatory networks and the evolution of animal body plans. *Science*, 311:796–800, 2006.
  - [109] Douglas H. Erwin and Eric H. Davidson. The evolution of hierarchical gene regulatory networks. *Nature Rev. Genetics*, 10:141–148, 2009.
  - [110] Marc Hetzel, Matthias Brock, Thorsten Selmer, Antonio J. Pierik, Bernard T. Golding, and Wolfgang Buckel. Acryloyl-CoA reductase from *Clostridium propionicum*: An enzyme complex of propionyl-CoA dehydrogenase and electron-transferring flavoprotein. *Eur. J. Biochem.*, 270:902–910, 2003.
  - [111] Gloria Herrmann, Elamparathi Jayamani, Galina Mai, and Wolfgang Buckel. Energy conservation via electron-transferring flavoprotein in anaerobic bacteria. *J. Bacteriol.*, 190:784–791, 2008.
  - [112] Wolfgang Buckel. Unusual dehydrations in anaerobic bacteria: considering ketyls (radical anions) as reactive intermediates in enzymatic reactions. *FEBS Letters*, 389(1):20 – 24, 1996.
  - [113] Megan J. Gruer, Peter J. Artymiuk, and John R. Guest. The aconitase family: three structural variations on a common theme. *Trends Biochem. Sci.*, 22:3–6, 1997.
  - [114] Miho Aoshima and Yasuo Igarashi. A novel oxalosuccinate-forming enzyme involved in the reductive carboxylation of 2-oxoglutarate in *Hydrogenobacter thermophilus* TK-6. *Mol. Microbiol.*, 62:748–759, 2006.
  - [115] Miho Aoshima and Yasuo Igarashi. Nondecarboxylating and decarboxylating isocitrate dehydrogenases: oxalosuccinate reductase as an ancestral form of isocitrate dehydrogenase. *J. Bacteriol.*, 190:2050–2055, 2008.
  - [116] Shelley D. Copley. Enzymes with extra talents: moonlighting functions and catalytic promiscuity. *Curr. Opin. Chem. Biol.*, 7:265–272, 2003.
  - [117] Elad Noor, Eran Eden, Ron Milo, and Uri Alon. Central carbon metabolism as a minimal biochemical walk between precursors for biomass and energy. *Mol. Cell*, 39:809–820, 2010.
  - [118] Harold J. Morowitz, Vijayasarathy Srinivasan, and Eric Smith. Ligand field theory and the origin of life as an emergent feature of the periodic table of elements. *Biol. Bull.*, 219:1–6, 2010.
  - [119] Michael J. Russell and Allan J. Hall. The onset and early evolution of life. *Geol. Soc. Am. Memoir*, 198:1–32, 2006.
  - [120] Juan C. Fontecilla-Camps, Patricia Amara, Christine Cavazza, Yvain Nicolet, and Anne Volbeda. Structure-function relationships of anaerobic gas-processing metalloenzymes. *Nature*, 460:814–822, 2009.
  - [121] Tzanko I. Doukov, Tina M. Iverson, Javier Seravalli, Stephen W. Ragsdale, and Catherine L. Drennan. A Ni-Fe-Cu center in a bifunctional carbon monoxide dehydrogenase/acetyl-CoA synthase. *Science*, 298:567–572, 2002.
  - [122] Javier Seravalli, Weiwei Gu, Annie Tam, Erick Strauss, Tadhg P. Begley, Stephen P. Cramer, and Stephen W. Ragsdale. Functional copper at the acetyl-coa synthase active site. *Proceedings of the National Academy of Sciences*, 100(7):3689–3694, 2003.
  - [123] Stephen W. Ragsdale. Nickel-based enzyme systems. *Journal of Biological Chemistry*, 284(28):18571–18575, 2009.
  - [124] Anne Volbeda, Marie-Helene Charon, Claudine Piras, E. Claude Hatchikian, Michel Frey, and Juan C. Fontecilla-Camps. Crystal structure of the nickel-iron hydrogenase from *Desulfovibrio gigas*. *Nature*, 373(6515):580–587, 1995.
  - [125] John W. Peters, William N. Lanzilotta, Brian J. Lemon, and Lance C. Seefeldt. X-ray crystal structure of the Fe-only hydrogenase (CPI) from *Clostridium pasteurianum* to 1.8 angstrom resolution. *Science*, 282(5395):1853–1858, 1998.
  - [126] MM Georgiadis, H Komiya, P Chakrabarti, D Woo, JJ Kornuc, and DC Rees. Crystallographic structure of the nitrogenase iron protein from *azotobacter vinelandii*. *Science*, 257(5077):1653–1659, 1992.
  - [127] J Kim and DC Rees. Structural models for the metal centers in the nitrogenase molybdenum-iron protein. *Science*, 257(5077):1677–1682, 1992.
  - [128] Kyle M. Lancaster, Michael Roemelt, Patrick Ettenhuber, Yilin Hu, Markus W. Ribbe, Frank Neese, Uwe Bergmann, and Serena DeBeer. X-ray emission spectroscopy evidences a central carbon in the nitrogenase iron-molybdenum cofactor. *Science*, 334(6058):974–977, 2011.
  - [129] Thomas Spatzal, Mge Aksoyoglu, Limei Zhang, Susana L. A. Andrade, Erik Schleicher, Stefan Weber, Douglas C. Rees, and Oliver Einsle. Evidence for interstitial carbon in nitrogenase FeMo cofactor. *Science*, 334(6058):940, 2011.
  - [130] A. Ricardo, M. A. Carrigan, A. N. Olcott, and S. A. Benner. Borate minerals stabilize ribose. *Science*,

- 303:196, 2004.
- [131] Arthur L. Weber. Sugars as the optimal biosynthetic carbon substrate of aqueous life throughout the universe. *Orig. Life Evol. Biosphere*, 30:33–43, 2000.
  - [132] Arthur L. Weber. Sugar model of the origin of life: Catalysis by amines and amino acid products. *Orig. Life Evol. Biosphere*, 31:71–86, 2001.
  - [133] Arthur L. Weber. Chemical constraints governing the origin of metabolism: The thermodynamic landscape of carbon group transformations under mild aqueous conditions. *Orig. Life Evol. Biosphere*, 32:333–357, 2002.
  - [134] Arthur L. Weber. Kinetics of organic transformations under mild aqueous conditions: Implications for the origin of life and its metabolism. *Orig. Life Evol. Biosphere*, 34:473–495, 2004.
  - [135] Eliane Fischer and Uwe Sauer. A novel metabolic cycle catalyzes glucose oxidation and anaplerosis in hungry *escherichia coli*. *Journal of Biological Chemistry*, 278(47):46446–46451, 2003.
  - [136] Dany J. V. Beste, Bhushan Bonde, Nathaniel Hawkins, Jane L. Ward, Michael H. Beale, Stephan Noack, Katharina Nöh, Nicholas J. Kruger, R. George Ratcliffe, and John Joe McFadden. 13c metabolic flux analysis identifies an unusual route for pyruvate dissimilation in mycobacteria which requires isocitrate lyase and carbon dioxide fixation. *PLoS Pathog*, 7(7):e1002091, 07 2011.
  - [137] Stephanie Markert, Cordelia Arndt, Horst Felbeck, Dörte Becher, Stefan M. Sievert, Michael Hügler, Dirk Albrecht, Julie Robidart, Shellie Bench, Robert A. Feldman, Michael Hecker, and Thomas Schweder. Physiological proteomics of the uncultured endosymbiont of *Riftia pachyptila*. *Science*, 315(5809):247–250, 2007.
  - [138] Goro Kikuchi. The glycine cleavage system: Composition, reaction mechanism, and physiological significance. *Mol. Cell. Biochem.*, 1:169–187, 1973.
  - [139] H. A. Barker and J. V. Beck. The fermentative decomposition of purines by *clostridium acidurici* and *clostridium cylindrosporium*. *Journal of Biological Chemistry*, 141(1):3–27, 1941.
  - [140] L J Waber and H G Wood. Mechanism of acetate synthesis from  $\text{CO}_2$  by *clostridium acidurici*. *Journal of Bacteriology*, 140(2):468–478, 1979.
  - [141] Kalin Vetsigian, Carl Woese, and Nigel Goldenfeld. Collective evolution and the genetic code. *Proc. Nat. Acad. Sci. USA*, 103:10696–10701, 2006.
  - [142] Pere Puigbo, Yuri Wolf, and Eugene Koonin. Search for a ‘tree of life’ in the thicket of the phylogenetic forest. *Journal of Biology*, 8, 2009.
  - [143] J. F. Kasting, D. H. Egger, and S. P. Raeburn. Mantle redox evolution and the oxidation state of the archaean atmosphere. *J. Geol.*, 101:245–257, 1993.
  - [144] James F. Kasting. Ups and downs of ancient oxygen. *Nature*, 443:643–644, 2006.
  - [145] Robert M. Hazen, Dominic Papineau, Wouter Bleeker, Robert T. Downs, John M. Ferry, Timothy J. McCoy, Dimitri A. Sverjensky, and Heixiong Yang. Mineral evolution. *Am. Mineralogist*, 93:1693–1720, 2008.
  - [146] D. Trail, E. B. Watson, and N. D. Tallby. The oxidation state of hadean magmas and implications for early earth’s atmosphere. *Nature*, 480:79–82, 2011.
  - [147] Roger Buick. When did oxygenic photosynthesis evolve? *Philosophical Transactions of the Royal Society B: Biological Sciences*, 363(1504):2731–2743, 2008.
  - [148] Lee R. Kump. The rise of atmospheric oxygen. *Nature*, 451(7176):277–278, 2008.
  - [149] William J. Brazelton and John A. Baross. Abundant transposases encoded by the metagenome of a hydrothermal chimney biofilm. *ISME Journal*, pages 1–5, 2009.
  - [150] William Joseph Brazelton. *Ecology of archaeal and bacterial biofilm communities at the Lost City hydrothermal field*. PhD thesis, University of Washington, 2010.
  - [151] Norman R. Pace. A molecular view of microbial diversity and the biosphere. *Science*, 276:734–740, 1997.
  - [152] Francesca D. Ciccarelli, Tobias Doerks, Christian von Mering, Christopher J. Creevey, Berend Snel, and Peer Bork. Toward automatic reconstruction of a highly resolved tree of life. *Science*, 311:1283–1287, 2006.
  - [153] William Martin and Michael J. Russell. On the origin of cells: An hypothesis for the evolutionary transitions from abiotic geochemistry to chemoautotrophic prokaryotes, and from prokaryotes to nucleated cells. *Philos. Trans. Roy. Soc. London*, 358B:27–85, 2003.
  - [154] Leslie E. Orgel. The implausibility of metabolic cycles on the early earth. *PLoS Biology*, 06:0005–0013, 2008.
  - [155] Ludmila Chistoserdova, Julia A. Vorholt, Rudolf K. Thauer, and Mary E. Lidstrom.  $\text{C}_1$  transfer enzymes and coenzymes linking methylotrophic bacteria and methanogenic archaea. *Science*, 281:99–102, 1998.
  - [156] Julia A. Vorholt, Ludmila Chistoserdova, Sergei M. Stolyar, Rudolf K. Thauer, and Mary E. Lidstrom. Distribution of tetrahydromethanopterin-dependent enzymes in methylotrophic bacteria and phylogeny of methenyl tetrahydromethanopterin cyclohydrolases. *J. Bacteriol.*, 181:5750–5757, 1999.
  - [157] L. Chistoserdova, M. G. Kalyuzhnaya, and M. E. Lidstrom. The expanding world of methylotrophic metabolism. *Annu. Rev. Microbiol.*, 63:477–499, 2009.
  - [158] Ludmila Chistoserdova. Modularity of methylotrophy, revisited. *Environmental Microbiology*, 13(10):2603–2622, 2011.
  - [159] Tobias J. Erb, Ivan A. Berg, Volker Brecht, Michael Mller, Georg Fuchs, and Birgit E. Alber. Synthesis of  $\text{c}_5$ -dicarboxylic acids from  $\text{c}_2$ -units involving crotonyl-coa carboxylase/reductase: The ethylmalonyl-coa pathway. *Proceedings of the National Academy of Sciences*, 104(25):10631–10636, 2007.
  - [160] Tobias J. Erb, Volker Brecht, Georg Fuchs, Michael Mller, and Birgit E. Alber. Carboxylation mechanism and stereochemistry of crotonyl-coa carboxylase/reductase, a carboxylating enoyl-thioester reductase. *Proceedings of the National Academy of Sciences*, 106(22):8871–8876, 2009.
  - [161] Thomas Handorf, Oliver Ebenhö, and Reinhart Heinrich. Expanding metabolic networks: scopes of compounds, robustness, and evolution. *J. Mol. Evol.*, 61:498–512, 2005.
  - [162] Jason Raymond and Daniel Segré. The effect of oxygen on biochemical networks and the evolution of complex life. *Science*, 311:1764–1767, 2006.
  - [163] Moritz Schütte, Alexander Skupin, Daniel Segré, and Oliver Ebenhö. Modeling the complex dynamics of enzyme-pathway coevolution. *Chaos*, 20:045115, 2010.
  - [164] David E. Graham and Robert H. White. Elucidation of methanogenic coenzyme biosyntheses: from spectroscopy to genomics. *Nat. Prod. Rep.*, 19:133–147, 2002.
  - [165] Tadhg P. Begley, Abhishek Chatterjee, Jeremiah W

- Hanes, Amrita Hazra, and Steven E Ealick. Cofactor biosynthesis still yielding fascinating new biological chemistry. *Current Opinion in Chemical Biology*, 12(2):118–125, 2008.
- [166] Christopher T. Jurgenson, Tadhg P. Begley, and Steven E. Ealick. The structural and biochemical foundations of thiamin biosynthesis. *Annual Review of Biochemistry*, 78(1):569–603, 2009.
- [167] Faping Huang, Charles Walter Bugg, and Michael Yarus. RNA-Catalyzed CoA, NAD, and FAD Synthesis from Phosphopantetheine, NMN, and FMN. *Biochemistry*, 39:15548–15555, 2000.
- [168] Jorge H. Crosa and Christopher T. Walsh. Genetics and assembly line enzymology of siderophore biosynthesis in bacteria. *Microbiology and Molecular Biology Reviews*, 66(2):223–249, 2002.
- [169] Alison Butler. Marine siderophores and microbial iron mobilization. *BioMetals*, 18:369–374, 2005.
- [170] Frank H. Westheimer. Why nature chose phosphates. *Science*, 235:1173–1178, 1987.
- [171] Harold B. White. Coenzymes as fossils of an earlier metabolic state. *J. Mol. Evol.*, 7:101–104, 1976.
- [172] Gregory A. Petsko and Dagmar Ringe. *Protein structure and function*. New Science Press, London, 2003.
- [173] Alex Gutteridge and Janet M. Thornton. Understanding nature’s catalytic toolkit. *Trends Biochem. Sci.*, 30:622–629, 2005.
- [174] Julia D. Fischer, Gemma L. Holliday, Syed A. Rahman, and Janet M. Thornton. The structures and physicochemical properties of organic cofactors in biocatalysis. *J. Mol. Biol.*, 403:803–824, 2010.
- [175] Claudia Andreini, Ivano Bertini, Gabriele Cavallaro, Gemma L. Holliday, and Janet M. Thornton. Metal ions in biological catalysis: from enzyme databases to general principles. *J. Biol. Inorg. Chem.*, 13:1205–1218, 2008.
- [176] Claudia Andreini, Ivano Bertini, Gabriele Cavallaro, Gemma L. Holliday, and Janet M. Thornton. MetalMACiE: a database of metals involved in biological catalysis. *Bioinformatics App. Note*, 25:2088–2089, 2009.
- [177] Stephen W. Ragsdale and Harland G. Wood. Enzymology of the acetyl-coa pathway of CO<sub>2</sub> fixation. *Critical Reviews in Biochemistry and Molecular Biology*, 26:261–300, 1991.
- [178] Stephen W. Ragsdale and Manoj Kumar. Nickel-containing carbon monoxide dehydrogenase/acetyl-coa synthase. *Chemical Reviews*, 96:2515–2540, 1996.
- [179] Stephen W. Ragsdale. Enzymology of the Wood-Ljungdahl pathway of acetogenesis. *Ann. N Y Acad. Sci.*, 1125:129–136, 2008.
- [180] Iris Fry. *The Emergence of Life on Earth: A Historical and Scientific Overview*. Rutgers U. Press, New Brunswick, N.J., 2000.
- [181] Raymond F. Gesteland, Thomas R. Cech, and John F. Atkins, editors. *The RNA World*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, third edition, 2006.
- [182] Shelley D. Copley, Eric Smith, and Harold J. Morowitz. The origin of the RNA world: Co-evolution of genes and metabolism. *Bioorganic Chemistry*, 35:430–443, 2007.
- [183] Michael Yarus. Getting past the RNA world: the initial Darwinian ancestor. In John Atkins, Ray Gesteland, and Tom Cech, editors, *Cold Spring Harbor Perspectives in Biology*, pages 1–8, Cold Spring Harbor, NY, 2011. Cold Spring Harbor Laboratory Press.
- [184] Robert E. MacKenzie. Biogenesis and interconversion of substituted tetrahydrofolates. In Raymond L. Blakely and Stephen J. Benkovic, editors, *Folates and Pterins, vol. 1: Chemistry and Biochemistry of Folates*, pages 255–306. John Wiley & Sons, New York, 1984.
- [185] Roland G. Kallen and William P. Jencks. The dissociation constants of tetrahydrofolic acid. *J. Biol. Chem.*, 241:5845–5850, 1966.
- [186] Patrick Stover and Verne Schirch. The metabolic role of leucovorin. *Trends in Biochemical Sciences*, 18(3):102–106, 1993.
- [187] Teng Huang and Verne Schirch. Mechanism for the coupling of atp hydrolysis to the conversion of 5-formyltetrahydrofolate to 5,10-methenyltetrahydrofolate. *Journal of Biological Chemistry*, 270(38):22296–22300, 1995.
- [188] M D Collins and D Jones. Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implication. *Microbiological Reviews*, 45(2):316–354, 1981.
- [189] Barbara Schoepp-Cothenet, Clément Lieutaud, Frauke Baymann, André Verméglio, Thorsten Friedrich, David M. Kramer, and Wolfgang Nitschke. Menaquinone as a pool quinone in a purple bacterium. *Proc. Nat. Acad. Sci. USA*, 106:8549–8554, 2005.
- [190] W. Nitschke, D.M. Kramer, A. Riedel, and U. Liebl. From naphtho- to benzoquinones - (r)evolutionary reorganizations of electron transfer chains. In P. Mathis, editor, *Photosynthesis: from Light to the Biosphere, vol. 1*, pages 945–950. Kluwer Academic Press, Dordrecht, 1995.
- [191] Michael J. Russell and A. J. Hall. The emergence of life from iron monosulphide bubbles at a submarine hydrothermal redox and pH front. *J. Geol. Soc. London*, 154:377–402, 1997.
- [192] Günter Wächtershäuser. Before enzymes and templates: A theory of surface metabolism. *Microbiol. Rev.*, 52:452–484, 1988.
- [193] Günter Wächtershäuser. Groundworks for an evolutionary biochemistry: the iron-sulphur world. *Prog. Biophys. Molec. Biol.*, 58:85–201, 1992.
- [194] Robert Shapiro. Small molecule interactions were central to the origin of life. *Quarterly Review of Biology*, 81:105–125, 2006.
- [195] Robert Shapiro. A simpler origin for life. *Scientific American*, Feb 12, 2007. (online).
- [196] Wolfgang Heinen and Anne Marie Lauwers. Organic sulfur compounds resulting from the interaction of iron sulfide, hydrogen sulfide and carbon dioxide in an anaerobic aqueous environment. *Origins of Life and Evolution of Biospheres*, 26:131–150, 1996. 10.1007/BF01809852.
- [197] G. D. Cody, N. Z. Boctor, R. M. Hazen, J. A. Brandeis, H. J. Morowitz, and Jr. Yoder, H. S. Geochemical roots of autotrophic carbon fixation: hydrothermal experiments in the system citric acid, H<sub>2</sub>O-(±FeS) (±NiS). *Geochimica et Cosmochimica Acta.*, 65:3557–3576, 2001.
- [198] K. Tazuya, C. Azumi, K. Yamada, and H. Kumaoka. Pyrimidine moiety of thiamin is biosynthesized from pyridoxine and histidine in *Saccharomyces cerevisiae*. *Biochem. Mol. Biol. Int.*, 36(4):883–888, Jul 1995.
- [199] Carlos F. III Barbas. Organocatalysis lost: modern

- chemistry, ancient chemistry, and an unseen biosynthetic apparatus. *Angew. Chemie Int. Ed.*, 47:42–47, 2008.
- [200] David W. C. MacMillan. The advent and development of organocatalysis. *Nature*, 455:304–308, 2008.
- [201] Powner, Matthew W. and Gerland, Béatrice and Sutherland, John D. Synthesis of activated pyrimidine ribonucleotides in prebiotically plausible conditions. *Nature*, 459:239–242, 2009.
- [202] Décout, Jean-Luc and Maurel, Marie-Christine. N6-substituted adenine derivatives and RNA primitive catalysts. *Orig. Life Evol. Biosphere*, 23:299–306, 1993.
- [203] Lubert Stryer. *Biochemistry*. Freeman, San Francisco, CA., second edition, 1981.
- [204] Tracy A. Lincoln and Gerald F. Joyce. Self-sustained replication of an RNA enzyme. *Science*, 323:1229–1232, 2009.
- [205] Shelley D. Copley, Eric Smith, and Harold J. Morowitz. A mechanism for the association of amino acids with their codons and the origin of the genetic code. *Proc. Nat. Acad. Sci. USA*, 102:4442–4447, 2005.
- [206] Daniel Segré and Doron Lancet. A statistical chemistry approach to the origin of life. *Chemtracts – Biochemistry and Molecular Biology*, 12:382–397, 1999.
- [207] Daniel Segré, Dafna Ben-Ali, and Doron Lancet. Compositional genomes: Prebiotic information transfer in mutually catalytic noncovalent assemblies. *Proc. Nat. Acad. Sci. USA*, 97:4112–4117, 2000.
- [208] Daniel Segré, Barak Shenhav, Ron Kafri, and Doron Lancet. The molecular roots of compositional inheritance. *J. Theor. Biol.*, 213:481–491, 2001.
- [209] Christian de Duve. *Blueprint for a cell*. Neil Patterson, Burlington, N. C., 1991.
- [210] Harry B. Gray. *Chemical bonds: an introduction to atomic and molecular structure*. University Science Press, Sausalito, CA, 1994.
- [211] George Wald. Life in the second and third periods: or why phosphorus and sulfur for high-energy bonds. In M. Kasha and B. Pullman, editors, *Horizons in biochemistry*, pages 127–142, New York, 1962. Academic Press.
- [212] Martin F. Hohmann-Marriott and Robert E. Blankenship. Evolution of photosynthesis. *Annu. Rev. Plant. Biol.*, 62:515–548, 2011.
- [213] Frederick Berkovitch, Yvain Nicolet, Jason T. Wan, Joseph T. Jarrett, and Catherine L. Drennan. Crystal structure of biotin synthase, an s-adenosylmethionine-dependent radical enzyme. *Science*, 303(5654):76–79, 2004.
- [214] Anne-Kristin Kaster, Johanna Moll, Kristian Parey, and Rudolf K. Thauer. Coupling of ferredoxin and heterodisulfide reduction via electron bifurcation in hydrogenotrophic methanogenic archaea. *Proc. Nat. Acad. Sci. USA*, 108:2981–2986, 2012.
- [215] W. E. Balche and R. S. Wolfe. Specificity and biological distribution of coenzyme M (2-mercaptoethanesulfonic acid). *J. Bacteriol.*, 137:256–263, 1979.
- [216] M. J. Danson. Central metabolism of the archaea. In M. Kates, D. J. Kushner, and A. T. Matheson, editors, *The biochemistry of archaea*, pages 1–24, Amsterdam, 1993. Elsevier.
- [217] Astrid Garhardt, Irfan Çinkaya, Dietmar Linder, Gjalts Hulsman, and Wolfgang Buckel. Fermentation of 4-aminobutyrate by *Clostridium aminobutyricum*: cloning of two genes involved in the formation and dehydration of 4-hydroxybutyryl-CoA. *Arch. Microbiol.*, 174:189–199, 2000.
- [218] Michael Schutz, Barbara Schoepp-Cothenet, Elisabeth Lojou, Mireille Woodstra, Doris Lexa, Pascale Tron, Alain Dolla, Marie-Claire Durand, Karl Otto Stetter, and Frauke Baymann. The naphthoquinol oxidizing cytochrome bc<sub>1</sub> complex of the hyperthermophilic knallgasbacterium aquifex aeolicus: Properties and phylogenetic relationships. *Biochemistry*, 42:10800–10808, 2003.
- [219] Fuli Li, Julia Hinderberger, Henning Seedorf, Jin Zhang, Wolfgang Buckel, and Rudolf K. Thauer. Coupled ferredoxin and crotonyl coenzyme A (CoA) reduction with NADH catalyzed by the butyryl-CoA dehydrogenase/Etf complex from *Clostridium kluyveri*. *J. Bacteriol.*, 190:843–850, 2008.
- [220] Shuning Wang, Haiyan Huang, Johanna Moll, and Rudolf K. Thauer. NADP<sup>+</sup> reduction with reduced ferredoxin and NADP<sup>+</sup> reduction with NADH are coupled via an electron-bifurcating enzyme complex in *Clostridium kluyveri*. *J. Bacteriol.*, 192:5115–5123, 2010.
- [221] William F. Martin. Hydrogen, metals, bifurcating electrons, and proton gradients: The early evolution of biological energy conservation. *FEBS Lett.*, 586:485–493, 2012.
- [222] Kesen Ma, Andrea Hutchins, Shi-Jean S. Sung, and Michael W. W. Adams. Pyruvate ferredoxin oxidoreductase from the hyperthermophilic archaeon, *Pyrococcus furiosus*, functions as a CoA-dependent pyruvate decarboxylase. *Proc. Nat. Acad. Sci. USA*, 94:9608–9613, 1997.
- [223] John E Cronan Jr. and Grover L Waldrop. Multi-subunit acetyl-coa carboxylases. *Progress in Lipid Research*, 41(5):407 – 435, 2002.
- [224] Hailong Zhang, Zhiru Yang, Yang Shen, and Liang Tong. Crystal structure of the carboxyltransferase domain of acetyl-coenzyme a carboxylase. *Science*, 299(5615):2064–2067, 2003.
- [225] Ronald Bentley and E. Haslam. The shikimate pathway a metabolic tree with many branches. *Critical Reviews in Biochemistry and Molecular Biology*, 25(5):307–384, 1990.
- [226] A. Kuki and P. G. Wolynes. Electron tunneling paths in proteins. *Science*, 236:1647–1652, 2000.
- [227] Roy A. Jensen. Enzyme recruitment in evolution of new function. *Annu. Rev. Microbiol.*, 30:409–425, 1976.
- [228] Dan S. Khersonsky, Olga Tawfik. Enzyme promiscuity: A mechanistic and evolutionary perspective. *Annu. Rev. Biochem.*, 79:471–505, 2010.
- [229] Juhan Kim, Jamie P. Kershner, Yehor Novikov, Richard K. Shoemaker, and Shelley D. Copley. Three serendipitous pathways in *E. coli* can bypass a block in pyridoxal-5'-phosphate synthesis. *Mol. Sys. Biol.*, 6:436:1–13, 2010.
- [230] Olga Khersonsky, Sergey Malitsky, Ilana Rogachev, and Dan S. Tawfik. Role of chemistry versus substrate binding in recruiting promiscuous enzyme functions. *Biochem.*, 50:2683–2690, 2011.
- [231] Dan K. Braithwaite and Junetsu Ito. Compilation, alignment, and phylogenetic relationships of DNA polymerases. *Nucl. Acids Res.*, 21:787–802, 1993.
- [232] Scott Bailey, Richard A. Wing, and Thomas A. Steitz.

- The Structure of *T. aquaticus* DNA Polymerase III Is Distinct from Eukaryotic Replicative DNA Polymerases. *Cell*, 126:893–904, 2006.
- [233] N. H. Horowitz. On the evolution of biochemical synthesis. *Proc. Nat. Acad. Sci. USA*, 31:153–157, 1945.
- [234] Francis Crick. Central dogma of molecular biology. *Nature*, 227:561–563, 1970.
- [235] Stephen Jay Gould. *The structure of evolutionary theory*. Harvard U. Press, Cambridge, Mass, 2002.
- [236] Edmund Beecher Wilson. *The cell in development and inheritance*. Macmilan, New York, third edition, 1925.
- [237] Poisot, Timothée and Bever, James D. and Nemri, Adnane and Thrall, Peter H. and Hochberg, Michael E. A conceptual framework for the evolution of ecological specialization. *Ecology Lett.*, doi:10.1111/j.1461-0248.2011.01645.x, 2011.
- [238] David L. Nelson and Michael M. Cox. *Lehninger Principles of Biochemistry*. W. H. Freeman, fourth edition, 2004.
- [239] Bruce Alberts. *Molecular biology of the cell*. Garland Science, New York, 4th edition, 2002.
- [240] Harold J. Morowitz. *Foundations of Bioenergetics*. Academic Press, New York, 1987.
- [241] Jean-Michel Claverie. Viruses take center stage in cellular evolution. *Genome Biol.*, 7:110:1–5, 2006.
- [242] Patrick Forterre. Defining life: the virus viewpoint. *Orig. Life Evol. Biosphere*, 40:151–160, 2010.
- [243] Anya K. Bershad, Miguel A. Fuentes, and David C. Krakauer. Developmental autonomy and somatic niche construction promotes robust cell fate decisions. *J. Theor. Biol.*, 254:408–416, 2008.
- [244] Deborah S. Kelley, John A. Baross, and John R. DeLaney. Volcanoes, fluids, and life at mid-ocean ridge spreading centers. *Annu. Rev. Earth Planet. Sci.*, 30:385–491, 2002.
- [245] H. Baltscheffsky, L.-V. von Stedingk, H.-W. Heldt, and M. Klingenberg. Inorganic pyrophosphate: formation in bacterial photophosphorylation. *Science*, 153:1120–1122, 1966.
- [246] Y. Yamagata, H. Watanabe, M. Saitoh, and T. Namba. Volcanic production of polyphosphates and its relevance to prebiotic evolution. *Nature*, 352:516–519, 1991.
- [247] Arthur Kornberg, Narayana N. Rao, and Dana Ault-Riché. Inorganic polyphosphate: A molecule of many functions. *Annu. Rev. Biochem.*, 68:89–125, 1999.
- [248] M. Baltscheffsky, A. Schultz, and H. Baltscheffsky.  $h^+$ -ppases: a tightly membrane-bound family. *FEBS Lett.*, 457:527–533, 1999.
- [249] Michael R. W. Brown and Arthur Kornberg. Inorganic polyphosphate in the origin and survival of species. *Proc. Nat. Acad. Sci. USA*, 101:16085–16087, 2004.
- [250] Juli Peretó, Purificación López-García, and David Moreira. Ancestral lipid biosynthesis and early membrane evolution. *Trends Biochem. Sci.*, 29:469–477, 2004.
- [251] M. M. Hanczyk, S. M. Fujikawa, and J. W. Szostak. Experimental models of primitive cellular compartments: Encapsulation, growth, and division. *Science*, 302:618–622, 2003.
- [252] Pier Luigi Luisi. *The emergence of life: from chemical origins to synthetic biology*. Cambridge U. Press, London, 2006.
- [253] Steven J. Freeland and Laurence D. Hurst. The genetic code is one in a million. *J. Mol. Evol.*, 47:238–248, 1998.
- [254] Robert D. Knight, Steven J. Freeland, and Laura F. Landweber. Selection, history and chemistry: the three faces of the genetic code. *Trends Biochem. Sci.*, 24:241–247, 1999.
- [255] Yi Lu and Stephen Freeland. On the evolution of the standard amino-acid alphabet. *Gen. Biol.*, 7:102:1–6, 2006.
- [256] Tze-Fei Wong. A co-evolution theory of the genetic code. *Proc. Nat. Acad. Sci. USA*, 72:1909–1912, 1975.
- [257] C. R. Woese, D. H. Dugre, W. C. Saxinger, and S. A. Dugre. The molecular basis for the genetic code. *Proc. Nat. Acad. Sci. USA*, 55:966–974, 1966.
- [258] Claude Berge. *Graphs and hypergraphs*. North-Holland, Amsterdam, rev. ed. edition, 1973.
- [259] Nathan D. Price, Iman Famili, Daniel A. Beard, and Bernhard O. Palsson. Extreme pathways and Kirchhoff’s second law. *Biophys. J.*, 83:2879–2882, 2002.
- [260] Iman Famili and Bernhard O. Palsson. Systemic metabolic reactions are obtained by singular value decomposition of genome-scale stoichiometric matrices. *J. Theor. Biol.*, 224:87–96, 2003.
- [261] Bernhard O. Palsson. *Systems Biology*. Cambridge U. Press, Cambridge, MA, 2006.
- [262] Adam M. Feist, Markus J. Herrgard, Ines Thiele, Jennie L. Reed, and Bernhard O. Palsson. Reconstruction of biochemical networks in microorganisms. *Nature Reviews Microbiol.*, 7:129–143, 2009.
- [263] Oktay Sinanoğlu. Theory of chemical reaction networks. all possible mechanisms or synthetic pathways with given number of reaction steps or species. *J. Amer. Chem. Soc.*, 97:2309–2320, 1975.
- [264] Oktay Sinanoğlu. On the algebraic construction of chemistry from quantum mechanics. a fundamental valency vector field defined on the euclidean 3-space and its relation to the hilbert space. *Theoret. Chim. Acta*, 65:243–248, 1984.
- [265] N. A. Sinitsyn, Nicolas Hengartner, and Ilya Nemenman. Adiabatic coarse-graining and simulations of stochastic biochemical networks. *Proc. Nat. Acad. Sci. USA*, 106:10546–10551, 2009.
- [266] S. L. Miller and D. Smith-Magowan. The thermodynamics of the krebs cycle and related compounds. *J. Phys. Chem. Ref. Data.*, 19:1049–1073, 1990.
- [267] J. D. Bernal, editor. *The Origin of Life*, London, 1967. Weidenfeld and Nicolson.